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EXPERIMENTAL CELL RESEARCH

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A COMPARISON OF THE ULTRAVIOLET ABSORPTION OF HAPLOID AND DIPLOID SALIVARY GLAND CHROMOSOMES[1,2]

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Received October 12, 1954

The present paper is concerned with two problems. One is to examine the justification for applying Lambert's law in the ultraviolet microspectrophotometry of chromosomes (see [14] and [29] for review). The other is the relationship between somatic pairing and growth of polytene chromosomes. The nature of the data is such that these two seemingly unrelated questions can best be considered together.

Triploid *Drosophila melanogaster*, having three instead of the usual two sets of chromosomes, furnished the material. One of the three homologous chromosomes often fails to pair with the other two for a portion of its length [27]. The two paired homologs are twisted about each other in typical rope-like fashion so that places can be found in which they are side by side, other places in which one is on top of the other. In each case, the extinction of the diploid can be compared with that of the haploid homolog in the same nucleus in a single photomicrograph.

The test of Lambert's law is made by comparing the diploid to haploid ratio when the paired chromosomes are adjacent to each other with the ratio found when the paired chromosomes are superimposed. Identical ratios would indicate conformance with the law, different ratios would indicate deviations from it.

The ratios in those cases in which the paired chromosomes lie side by side constitute the first explicit test of the effect of pairing on the growth of polytene chromosomes. It is already known that homologous haploid bands have essentially the same extinction [11]. In the absence of an effect of pairing,

[1] Supported by a research grant (C1613) from the National Cancer Institute of the National Institutes of Health, Public Health Service, and by an institutional grant from the American Cancer Society.

[2] Presented in part at the meetings of the Histochemical Society at Chicago, April, 1953. (*J. Histochem. and Cytochem.* **1**, 398 (1953) abst.)

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all three homologs in a triploid nucleus would be expected to give the same result, i.e., the diploid pair would have just twice the haploid value. If pairing were, for example, to inhibit or enhance the rate of growth, the diploid would have, respectively, more or less than twice the total extinction found for its haploid homolog.

The measurements to follow show that a two to one ratio obtains regardless of the orientation of the diploid strands.

MATERIALS AND METHODS

Biological material.—The triploid individuals used in these experiments came from mothers treated with colchicine during the first larval instar. After this treatment, a few of the females yield exclusively polyploid progeny (triploid females and intersexes [28]).

Since both parents of the triploids arose from a highly inbred strain of *Drosophila melanogaster* (Oregon R, 100 generations of inbreeding), all three sets of chromosomes of the triploid are presumed to be identical.

Later in the work (see F and G, Table I), use was made of larvae containing three X chromosomes, with only two sets of autosomes. These were progeny of triploid mothers derived from colchicine treatment. Here, also, the three X chromosomes are equivalent genetically.

Experimental larvae were raised at 25°C on a cornmeal-molasses-agar medium seeded with live baker's yeast. They were collected near the end of the migratory period that precedes puparium formation.

Preparation of material.—Some modifications of the usual salivary gland smear technique, recently developed in our laboratory [27] have increased the frequency with which suitable chromosome figures are obtained on quartz slides. Salivary glands were dissected in insect ringer's and fixed in fifty per cent aqueous acetic acid for three to five minutes at 20–22°C. They were then pressed in a drop of fixative between a quartz slide and a siliconed (G.E. Dri-Film 9987) glass cover slip. The silicone greatly facilitates the spreading of the chromosomes and makes it possible to remove the cover slip with a minimum loss of chromosomes from the slide.

Gentle tapping with a needle separated the cells, then disrupted the cytoplasm. A few raps with a rubber hammer disrupted nuclei and partly dispersed the cytoplasm, a process completed by further tapping with the needle. The smear was completed by firmly rolling a thumb over it while it was covered with a piece of absorbent paper to take up the expressed fixative. The slide was examined with a phase microscope to determine whether the chromosome composition was the desired one.

The slide was then frozen on block of dry-ice, and the cover slip was removed with a cold razor blade. A drop of lanthanum in glycerine (five per cent saturated aqueous lanthanum acetate, ninety five per cent glycerine) was added and covered with a quartz cover slip. After storage of the preparation overnight (4–8°C), excess mounting fluid was expressed (taking with it much of the remaining cytoplasm) and the cover slip was sealed with a paraffin-gum mastic mixture. The slides were again exam-

TABLE I

Total extinction in square micra of unpaired homologous haploid and diploid chromosome sections in triploid nuclei and in nuclei trisomic for the X-chromosome in the salivary glands of *Drosophila melanogaster*.

Plate letter	Chromosome arm section	Extinction at 257 mμ			Extinction at 275 mμ		
		Diploid	Haploid	Deviation[1] (per cent)	Diploid	Haploid	Deviation[1] (per cent)
A	3L 63F – 65D	10.80	5.95	+ 6.6	12.10	6.34	+ 3.1
B	2R 54A – 58D	10.90	5.41	– 0.5	11.30	5.86	+ 2.4
C	2R 54A – 56F	13.10	5.94	– 6.5	11.00	5.15	– 5.0
D	2R 52B – 52E	2.86	1.45	+ 0.7	1.93	0.98	+ 1.2
E	2L 21C – 22C	9.93	5.20	+ 3.9	6.34	3.32	+ 3.1
	22A[2]	2.70	1.35	± 0.0	1.61	0.86	+ 4.7
	21C[2]	1.64	0.83	+ 1.2	1.04	0.55	+ 3.6
F	X 3A	2.06	1.01	– 1.2	1.39	0.60	– 9.6
	3B	1.22	0.43	–21.9	0.91	0.40	– 7.6
	3C	2.27	1.18	+ 2.6	1.31	0.70	+ 4.2
	3D	3.17	1.51	– 3.1	–	–	–
	3A – 3D[3]	(8.87)	(4.20)	(– 3.6)	(5.66)	(2.79)	(– 0.8)
G	X 3A	1.62	0.81	– 0.7	1.25	0.76	+11
	3B	0.43	0.21	– 2.1	0.30	0.20	+20
	3C	1.72	1.04	+12.6	1.38	0.96	+23
	3A – 3C[3]	(3.78)	(2.08)	(+ 6.5)	(2.94)	(1.91)	(+18)
	Total[4]	64.42	32.32	+ 0.2	48.93[4]	24.76[4]	+ 0.8

[1] Deviation of haploid from one third of the sum of haploid and diploid.
[2] Run at slower scanning speed than the whole section, 21C–22C.
[3] Planimetered separately from subsections, but from same records.
[4] Excluding section totals (in parentheses). The measurements from G at 275 mμ are also excluded; the large excess in haploid is probably the result of a faulty photographic plate. Other data, including Fig. 9 and measurements at 275 mμ after the photographs for Fig. 9 had been taken, are in much closer agreement with a 2 : 1 ratio.

ined with the phase microscope to select chromosomes in which one homolog had failed to pair with the other two and the field was clear of foreign matter.

Photomicrographic apparatus.—The optical system is diagrammed in Fig. 1. The light source and monochromator (ABC) were identical to the ultraviolet illuminator described by Köhler [18] and used by Caspersson [7]. Stray light was absorbed by a Bäckström [1, 16] type filter in a quartz cell (E). The photomicrographic apparatus on which items D to O were mounted was a Leitz Panphot stand to which quartz optics had been fitted. Three glycerine immersion monochromats were available during the course of the work: a Zeiss 2.5 mm. 0.85 N.A.[1] and a Cooke 2 mm. 1.25 N.A. corrected for 2750 Å; and a Cooke 1.25 N.A. corrected for 2536 Å. Magnification at

[1] Courtesy of Professor C. W. Metz, University of Pennsylvania.

the plate (10 × ocular, 38 cm bellows) was 1320 diameters with the Zeiss lens, 1500 diameters with the Cooke lenses. A 1.25 N.A. Cooke quartz glycerine immersion condenser, used with all objectives, was stopped down with a 7 mm diaphragm (N.A. about 0.5).

Photographic methods.—Eastman spectrographic plates, type V-O, developed in a high contrast developer [30] were employed for most of the work. Process plates

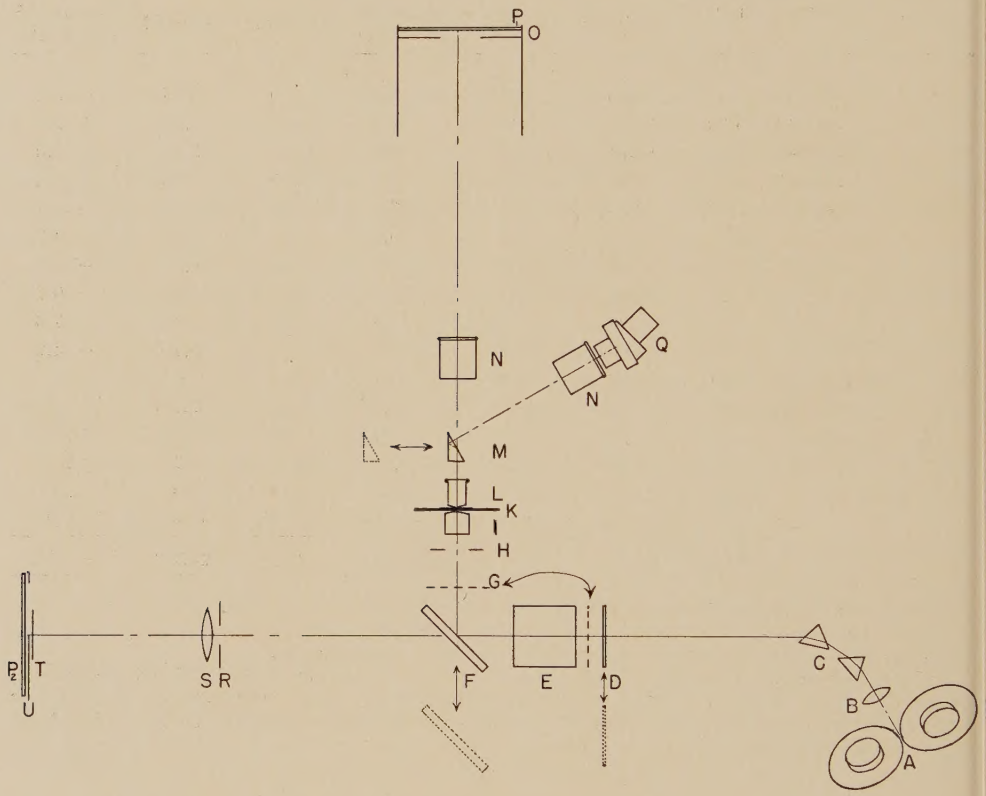


Fig. 1. Diagram of the optical system (not to scale). ABC, monochromatic ultra-violet illuminator after Köhler, of which the rotating electrodes, A, the collimating lens, B, and two water-filled, quartz-faced prisms, C, are shown; D, removable fluorescent screen for aiding the adjustment of light from ABC; E, nickel and cobalt filter after Bäckström; F, removable aluminized front-surface mirror; G, removable 200 mesh per inch bolting silk filter for reducing the intensity of extremely bright lines; H, diaphragm for I; I, quartz condenser; K, object to be photographed, between quartz slide and cover slip; L, quartz monochromat (see text); M, removable prism for deflecting the image from L to the side-arm accessories, N and Q; N, quartz ocular; Q, fluorescent finder for finding and focusing by ultra-violet light; O, reflex housing with mask and provision for taking several exposures on a single plate; P_1, plate in position for photomicrograph; R, diaphragm for S; S, 15 mm focal length lens for decreasing the intensity of light from ABC at T when F has been removed; T, rotating logarithmic step sector; U, mask; P_2, plate in position for calibration exposure through T after photomicrograph has been taken on it in position P_1.

developed in the same developer for one and one half minutes were used for the last two preparations. The developer was agitated by moving the edge of a piece of plastic (homalite) back and forth over the plate at a clearance of about 0.05 inches about once every two seconds [17, 19]. Plates were calibrated in one of two ways. The first, which we shall call method I, is similar to that first described by Caspersson ([7], see also [21]). Portions of sample plates were exposed at known bellows extensions (and therefore known relative intensities) in the ultraviolet microscope. One portion of each plate was then exposed through an optical step wedge to light from a battery operated tungsten lamp under precise voltage control. The wedge was made of rhodium evaporated onto quartz and therefore could be used as well in the ultraviolet [17, 32]. Since the rhodium film was not neutral and the spacing of the steps was rather large, it was inconvenient to use the wedge for direct calibration. However, it was used to increase the range of light intensities to which a single plate was exposed at each bellows extension. The calibration curve for the wave length and emulsion number was then constructed and a standard curve for the wedge in the visible determined. Subsequently, a portion of each measuring plate was exposed to the same visible light source through the same wedge for a control on photographic processing and plate variability. Such plates were used for serial numbers A to E, Table I.

In the second method (II), no visible light was used. By removing mirror F, a portion of each plate was exposed to the light from the monochromator, ABC, through a rotating logarithmic step sector, T, immediately after and for the same time as the exposure for the photomicrograph. Difficulties of obtaining an evenly illuminated clear space next to the chromosome and in a place suitable for a sector (or wedge) to be included in the image plane of the microscope were thus avoided. The light intensity at the sector was adjusted to produce a plate density slightly greater than the background density of the photomicrograph. Plates for serial numbers F and G were calibrated in this way.

Theoretical objections have been raised against the use of a sector for the calibration of photographic emulsions exposed to intermittent light sources; failure of the reciprocity law, intermittency effect and the possibility of interference between the spark and sector frequencies [19, 30]. These were tested in our apparatus by comparing calibration curves obtained by method II with exposures made on the same plates by method I. Since they agreed, it follows that within the range of exposures we use, the sector introduces no serious error.

The precision with which the calibration curve must be known depends on the use to be made of the measurement. For comparisons of ratios of extinctions on a single plate, as in these data, it is only necessary that the measure of plate blackening (deflection of the recorder pen) be directly proportional to the logarithm of the intensity of the radiant energy reaching the plate. Even large errors in the experimentally determined proportionality constant would not affect the observed ratios, but would result in errors in the individual values obtained for the total extinctions; in these data, between the different regions and between extinctions of the same region at different wave lengths.

The major advantage of method II lies in the fact that the calibrating exposures are made on the same plate as the experimental exposure. Secular variations in the calibration curves obtained on plates carrying the same emulsion number introduced

a considerable error by our method I. Therefore, all of the more recent work in this laboratory has made use of method II.

Densitometric measurements were carried out with a Jarrel-Ash recording microphotometer which indicated per cent transmission on a linear scale. Where the exposure was such that the calibration showed the logarithm of the light intensity to be proportional to per cent transmission, the photometer was used directly. When the exposures were such that this relation did not hold, use was made of an electronic function transformation system[1] introduced between the photometer and the recorder. The output of the microphotometer was transformed according to the calibration curve of the plate to be linear in extinction at the recorder.

The determination of the total extinction.—Our procedure for determining the total extinction of a cytological structure is essentially similar to that used by Caspersson and Schultz [9, 10, 12, 25, 21] and by Walker and Davies [33, 14]. There are differences in detail that add some convenience and speed; such are the design of the microdensitometer and the second method of plate calibration. The following example explains the procedure in more detail than is found in the previously published accounts.

The photomicrograph in Fig. 2 is an enlargement from the plate on which the extinction of Fig. G in Table I was measured at 257 millimicra. The triplo-X chromosome is seen to bifurcate into a haploid strand to the right and a diploid strand to the left. The branching point is in sub-section 3D (following Bridges' [4] standard salivary gland chromosome map). Sub-sections A, B and C, in which we were interested, are included in the failure of pairing and have been identified by the appropriate letters on the photograph.

There are four steps necessary to complete the measurement. They will be described in the order in which they are performed.

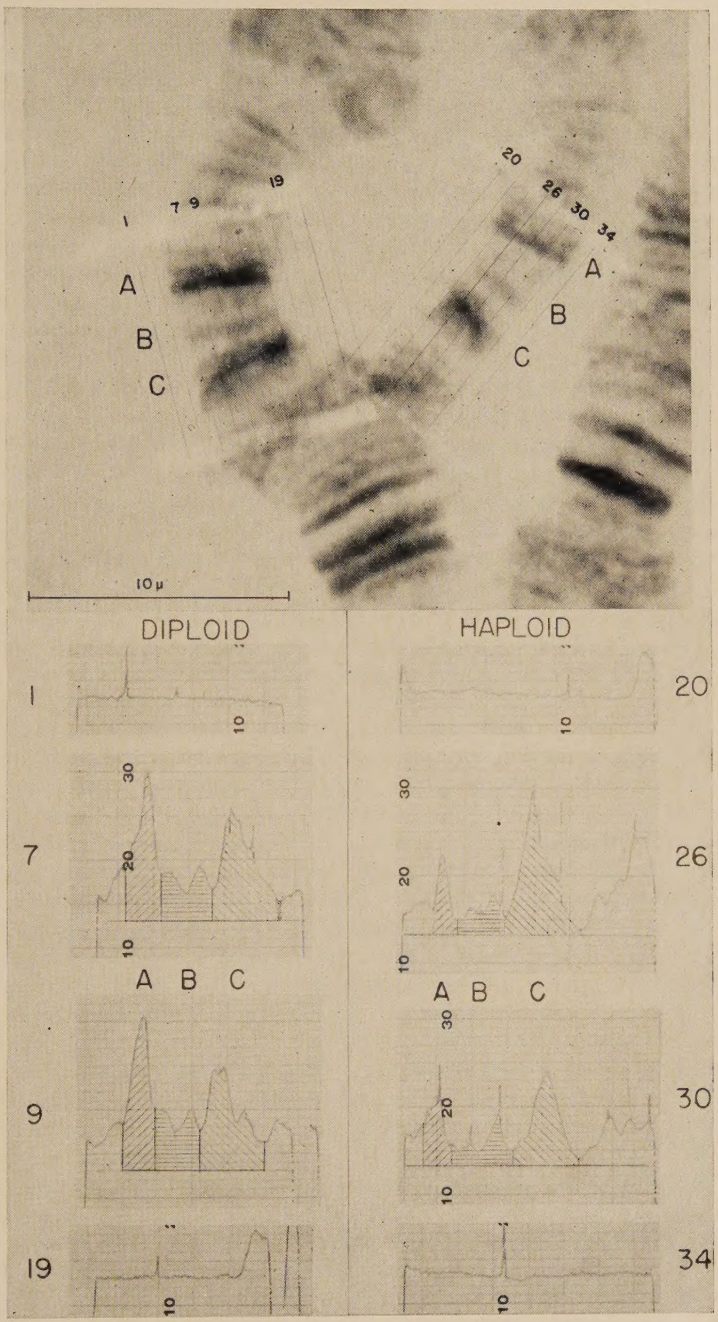
1. Identification and delimitation of the area to be measured on the plate. The marking was done on the back of the plate with a glass marking pencil, and shows in the figure as broad white lines across the two unpaired chromosomes.

2. Scanning the demarcated area and the calibrating sector with the recording

[1] Designed and constructed by Mr. Alan Bloch and the Audio Instrument Company, New York, whose understanding cooperation is greatly appreciated.

Fig. 2. Above: ultra-violet photomicrograph (257 millimicra) of part of X chromosome G, Table I; Below: densitometric records from the same negative.

The three X-chromosomes are paired at the bottom of the photomicrograph; the failure of pairing begins in sub-section 3D. Sub-sections A, B and C of section 3, included in the unpaired region, are indicated on the figure. The plate was scanned along paths represented by the dark lines, some of which are numbered to correspond with the numbers of the records below. The x-axis in the records is distance along a run, the y-axis is proportional to plate transmission and to extinction. The area between the incident light level and the light level transmitted by the chromosome is proportional to the total extinction of the strip of chromosome represented by the run. The shaded portions correspond roughly to subsections A, B, and C as above. The areas actually measured were about 16 times the size in this figure. ("A" includes approximately bands A1 through A6; "B" includes approximately bands A7 through B4; "C" includes approximately bands C1 through C9). See text for further details. (Cooke 2 mm monochromat 2536 Å, N.A. 1.25; 257 mmμ cadmium line).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19
20
26
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34
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DIPLOID
HAPLOID
10
20
30
A B C

microphotometer. The sector was not included in the print because of lack of space. The scanning proceeds in a series of parallel lines perpendicular to the grease pencil lines and spaced one half millimeter apart. Black lines have been drawn on the print in Fig. 2 to represent half of the runs actually made; i.e., the plate was scanned along each of the lines drawn and half-way between them as well. The beam of light used for scanning covers an area below the resolving power of the microscope (0.125 by 0.025 mm at the plate, 0.083 by 0.0017 micra at the object, or about one by two half-tone spots in a light grey portion of the reproduction here.

3. Collation of the photometer records with the chromosome image and preparation for planimetry. The horizontal axis on the records (Fig. 2, lower half) is distance along the run, the vertical axis is proportional to plate blackening, in this case to per cent transmission. The particular region to be measured is identified on each of the runs and its limits demarcated by suitably placed ordinates. Care must be taken that the same bands are included in each run and in each homolog. The shorter the section being measured, the greater the relative effect of an error in identification. Such errors are particularly to be guarded against when the two homologs are not extended to the same degree. In this example, the haploid is "stretched" more than is the diploid. Most of the regions reported on here are long enough to reduce the possible effects of uncertainty in identification to a minimum.

The calibrating sector had shown that the per cent transmission of this plate was directly proportional to the logarithm of the relative intensity of the light reaching the plate within the range of plate blackening covered by the image of the chromosome. Therefore, the difference between any two ordinates within that range is proportional to the logarithm of the ratio of two light intensities, i.e., to extinction.

4. Planimetry and conversion to convenient units. The incident light as given by records number 1, 19, 20 and 34 has been drawn on the other runs which included the image of the chromosome. The position of the grease pencil marks is indicated by a downward excursion of the recorder pen at each end of a run. Between these, the parts of each trace corresponding to the bands to be measured have been marked. The areas between them and the incident level have been cross-hatched for purposes of illustration. The difference between the incident light level and any point on the trace over a chromosome is, according to the calibration, proportional to the extinction of the chromosome at a corresponding point. The sum of the extinctions at all points along a run is proportional to the cross-hatched area. This area, converted to the appropriate units, is the total extinction of the strip of chromosome lying within one half the distance to the scanning run on either side. Our run interval of one half millimeter corresponds to one third of a micron at the chromosome itself, or about three times the resolving power of the microscope. The assumption that the extinction indicated by a scanning run is the average extinction over a strip that wide is not expected to introduce serious errors. All thirty runs were made, the records were prepared in the way just indicated, and the cross-hatched areas were measured with a planimeter. The sum of all such areas on runs over, e.g., section A of the diploid chromosome multiplied by the appropriate instrument constant and by the slope of the calibration curve for this plate is the number recorded in Table I under "diploid" opposite "G" as the total extinction of subsection 3A. The area for A, B and C together was planimetered separately from each of its parts.

The instrument constant was so taken that the total extinction could be expressed

in convenient units. It has the dimensions of area multiplied by the dimensionless extinction. We have chosen the square micron as the unit.

Reliability of densitometry.—Densitometry is quite reproducible when the same area of a plate is run repeatedly. As an example, we may cite the repetition of the analysis of this same chromosome at 275 millimicra. The plate from which the measurement was made was completely reanalyzed one month after the original measurement. Total extinctions of the diploid parts agreed with the earlier ones, differing by less than one and three quarters per cent; the haploid values agreed within three and one half per cent with the exception of sub-section 3B (G_2 in Table I) which was 9.4 per cent higher in the second running. The uncertainties in delimiting the boundaries of this very short section minimize the significance of this deviation. It was obvious that the disagreement of the observed extinctions with those expected for a 2:1 ratio (see Table I) was not the result of an error in densitometry.

To get an idea of the orders of magnitude in these measurements, let us assume that nucleic acids are responsible for ninety per cent of the total extinction of subsection 3C. Then, using as an extinction coefficient the value of 2.58 for one micromicrogram (10^{-12} gm) per square micron [5], we would find approximately 1.3 micromicrograms of nucleic acids in the haploid. Similar assumptions lead to a value of 16 micromicrograms of nucleic acids in the largest diploid measured (C of Table I). It need not be stressed that these rough calculations can only be useful as indicators of order of magnitude. The extinction coefficients of the chromosome constituents (of which DNA is only one) under conditions prevailing in these preparations have not been determined. Extrapolation from absorption data obtained in dilute solutions to the relatively high concentrations encountered here oversteps the range within which the Beer-Lambert law is known to hold [5].

Estimates of length and area.—Having obtained the total extinction, our analysis is completed by measuring the length of the cross-hatched portion of each record. This measurement we shall call the length of the run. It may be used to obtain two additional types of information about the chromosome, namely, the length and the area of the optical section measured.

The length of the longest run, expressed in micra, is used as a measure of the length of the chromosome section. It is not an accurate measure, especially if the chromosome is curved as in Figs. 3, 4, 5 and 7, but it can be used to compare diploid with haploid in all of our cases.

The sum of the lengths of all of the runs is a measure of the area of the optical section of the chromosome. After correction for run interval, it can be converted into square micra.

Both of these measures as well as the ratios between the diploid and haploid values as measured from the plates taken at 275 millimicra are recorded in Table III. The last column in the same table shows the areas corrected for differences in length as explained in detail in the next section.

RESULTS

Quantitative equality of homologs.—All figures judged to be suitable for photometry were measured from photographs taken with the cadmium lines

at 257 and 275 millimicra. The total extinctions, given in Table I, are distributed around the predicted equality of all three homologs in a nucleus. Expectations have been calculated giving equal weight to each of the three chromosomes involved; deviations are those of the unpaired haploid from the mean of all three.

TABLE II

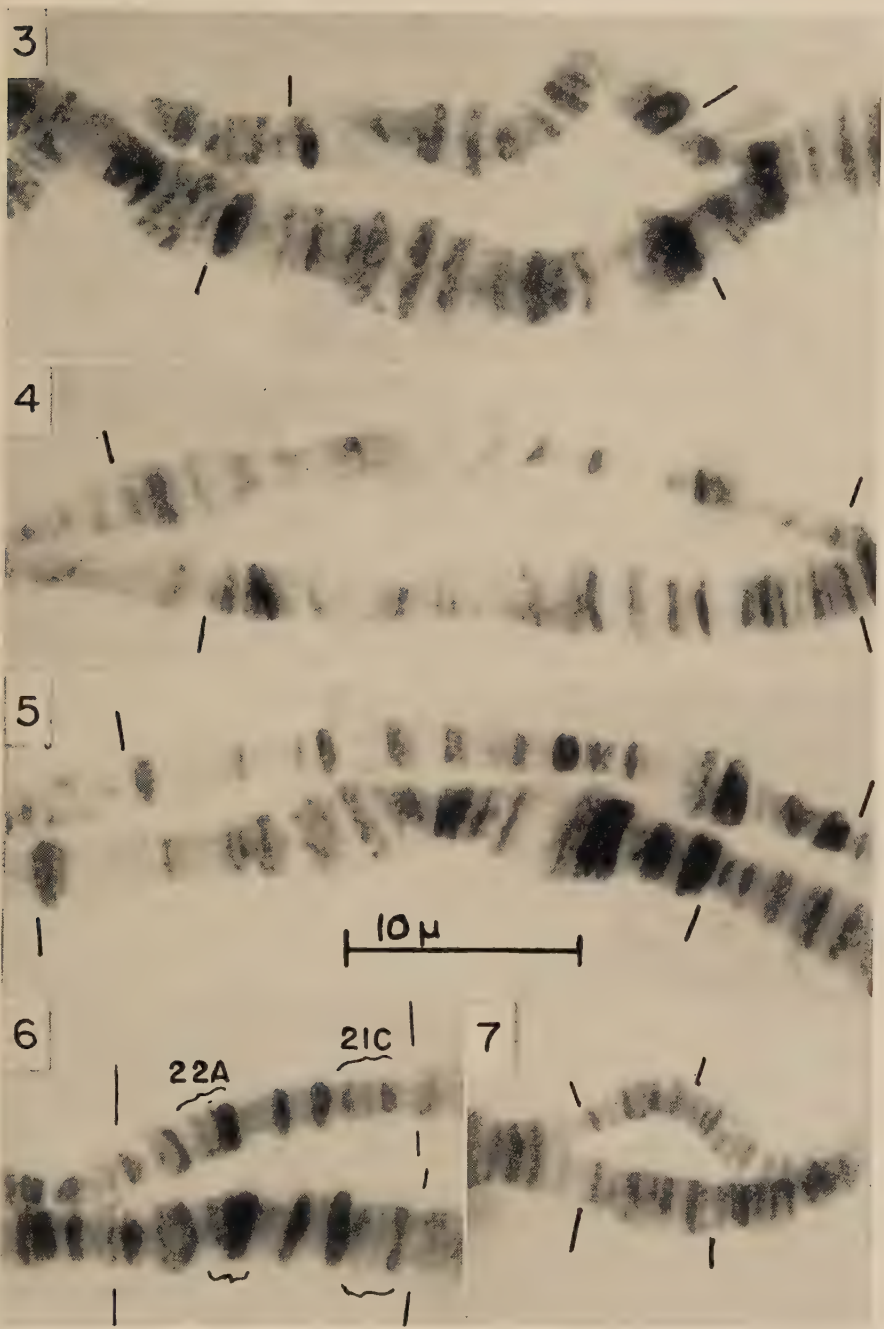
Regression analysis of data in Table I.

Dependent variate	Independent variate	Wave length	(b) Regression coefficient	Standard deviation of (b)	*P* that origin is on line	N
Diploid	Haploid	257 mμ	2.00	$\pm$0.06	.9	16
Diploid	Haploid	275 mμ	1.99	$\pm$0.05	.5 – .7	15
Haploid	Diploid	257 mμ	0.49	$\pm$0.02	.9	16
Haploid	Diploid	275 mμ	0.50	$\pm$0.01	.3 – .5	15

The deviations at 257 millimicra, near the maximum specific extinction of the nucleic acids, nearly cancelled each other in the totals, leaving a very good fit to the expected ratio. The data have been treated statistically in two ways. The regression of haploid on diploid is shown in Table II to have a slope of 0.49 ± 0.02, not significantly different from 0.50; likewise, the reciprocal slope is not significantly different from 2.00. Both lines pass through the origin, showing that there is no significant excess in extinction associated with the paired or the unpaired state.

The linear regression is somewhat biased in this case because the extinctions cover a sixty-fold range, and the deviations tend to be proportional to the absolute value of the extinction (see Table I and Fig. 8). The agreement is, however, equally good if a logarithmic transformation is used, thereby weighting deviations according to the value of total extinction. The mean of the differences between the logarithms of haploid and diploid is 0.310 with a standard error of ± 0.014, not significantly different from the expected

Fig. 3–7. Unpaired portions of salivary gland chromosomes of triploid *Drosophila melanogaster* in which one chromosome (the upper one in each figure) has failed to pair with the other two (the lower, diploid chromosome in each figure). The total extinction of the parts included between the black marks adjacent to the chromosomes may be found in Table I. (Zeiss 2.6 mm monochromat 2750 Å, N.A. 0.85; Cooke quartz condenser; 257 mmμ cadmium emission line; reproduced at about 3000 diameters.)
Fig. 3. Unpairing C in Tables I and III; Fig. 4. Unpairing A in Tables I and III; Fig. 5. Unpairing B in Tables I and III; Fig. 6. Unpairing E in Tables I and III; Fig. 7. Unpairing D in Tables I and III.


3
4
5
10 μ
6
21C
22A
7

0.301. The standard deviation of the population of differences is ± 0.05 log units. For the ratios themselves, this standard deviation corresponds to a coefficient of variation (v) of about 12 per cent.

Of these chromosomes, all but one was measured also at 275 millimicra, near the maximum specific extinction of the aromatic amino acid residues of proteins. The results, listed in columns 6 and 7 of Table I and included in

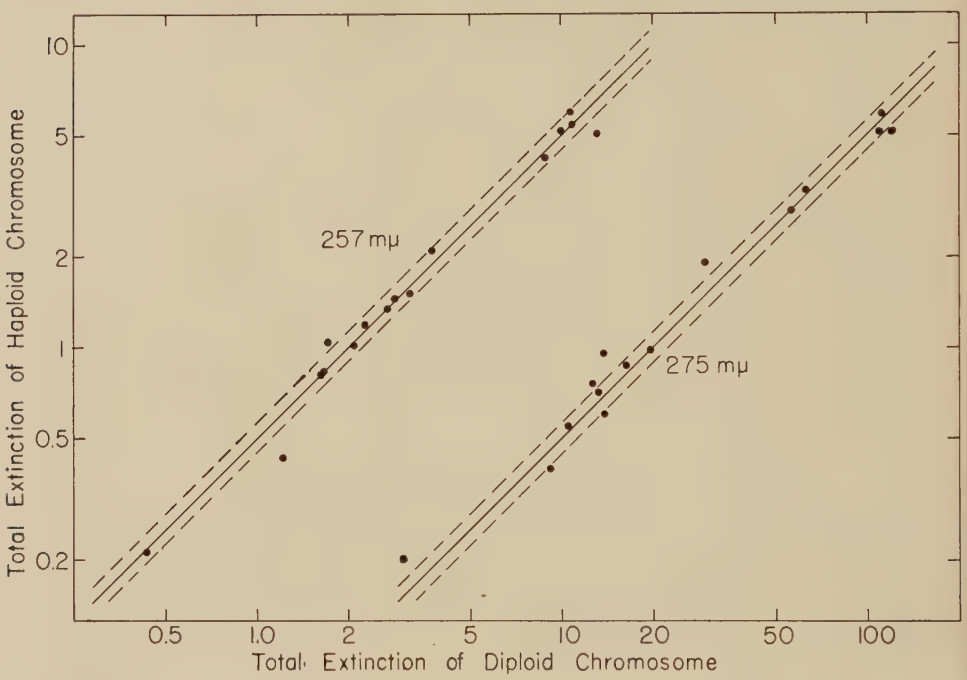


Fig. 8. Log log plot of the data in Table I. The diploid values at 275 mmμ have been moved one log unit to the right to separate the two sets of data. The solid lines are for diploid equal to twice haploid, the dashed lines are at plus and minus 10 per cent of the solid line. See Table II for statistical agreement of the data with these lines.

Fig. 8, tell the same story, namely, that the total extinction at this wave length as well is proportional to the number of chromosomes. The regression constants in Table II show, as before, no statistical disagreement with the expectation. The totals show a net deviation of $+0.8$ per cent. Statistical treatment of the differences between the logarithms gives a mean of 0.30 with a standard error of ± 0.10 and a standard deviation of ± 0.03 (± 8 per cent of the measured ratio). If the measurements from plate G275 are included in the calculations, the mean becomes 0.274 ± 0.016, still not sig-

nificantly different from an expected log 2, and the standard deviation of the whole set of data is correspondingly higher (± 0.06 log units, equivalent to $v = \pm 15$ per cent in actual ratio).

Qualitative similarity of homologs.—Statistical equality of both chromosomes at 257 and at 275 millimicra implies that, as one would expect, homologous chromosome sections in a nucleus have the same constitution with regard to substances absorbing at these two wave lengths. We have examined this corollary in two ways. First, if the three chromosomes are identical, then the ratio of the extinction at 257 to that at 275 should be the same for both members of a pair. That turned out to be the case. The ratios in diploid and haploid within the same nucleus are statistically identical ($P > 0.3$) although the ratios observed in different preparations are different from each other).

The second check on the qualitative equivalence of the two homologs was made by comparing their extinctions at other wave lengths in the ultraviolet. This was done for four of the preparations, and four of the sets of data so obtained are presented in graphic form in Fig. 9. The shape of the absorption curves so obtained as indicated by the lines joining the points is, within the limits of the method, that which would be expected of a nucleoprotein, and does not disagree with the more precise curves obtained from euchromatic portions of salivary gland chromosomes by Caspersson [8]. Deviations from the two-to-one ratio become larger at wave lengths far from the two we have been discussing. These are interpreted as most likely the result of defects in the optical system rather than differences between the two homologs.

Only the two monochromats corrected at 275 and at 257 millimicra were available for these measurements. We expect the correspondence between the intensity relations in the object and those in the image to be best at those wave lengths. Spherical aberration, coma and glare contribute to the deterioration of the image in other parts of the spectrum [17, 14]. The errors so introduced into these measurements are of two kinds. First, the demarcation of the chromosome bands is not as precise in the poorer images. This, along with the low contrast at the longer wave lengths, introduces random deviations from the true values. Secondly, the increase in glare which accompanies spherical aberration would be expected to have a larger effect on the smaller of two objects. The consistently low haploid values found at the shorter wave lengths are in conformity with this expectation. It might be possible to use this system for testing methods of reducing errors of this type.

These data, together with the more extensive ones at the two critical

wave lengths, are therefore in reasonable agreement with the expected similarity of all three homologs.

The Beer-Lambert law in chromosomes.—The proportionality that we find between chromosome mass and total extinction implies that the Beer-Lambert law applies over the range of differences in concentration and thickness between homologous parts of these haploid and diploid chromosomes. There

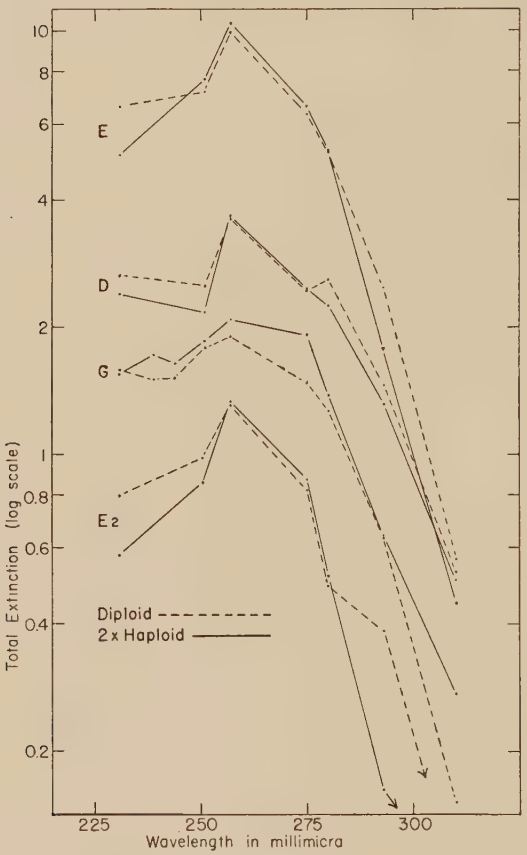


Fig. 9. Total extinction of four unpaired chromosome regions at various wave lengths in the ultra-violet. The extinction axis is logarithmic to permit direct comparison of ratios of values at different wave lengths. Some of the "curves" have been moved downward to avoid overlapping. The letters refer to the serial letters as given in the first column of Table I. The wave lengths used are those available from cadmium, magnesium and nickel electrodes (231, 239, 244, 251, 257, 275, 280, 293 and 310 millimicra).

is no *a priori* reason to expect a difference in volume between paired and unpaired homologs (and therefore, a difference in effective concentration), but variations in thickness are expected to be greater in the diploid than in the haploid. These may be detected as differences in the area of the image. If the diploid chromosomes lie side by side, then they will cover about twice the area covered by the haploid and be about the same thickness. If the paired

TABLE III

Areas and lengths of homologous diploid and haploid chromosome segments in salivary gland nuclei of *Drosophila melanogaster*, measured from photomicrographs taken at 275 millimicra.

Plate letter[1]	Area[2] (square micra)			Length[2] (micra)			Est. ratio of widths[3] (col. 4/col. 7)
	Diploid	Haploid	Diploid / Haploid	Diploid	Haploid	Diploid / Haploid	
A	118.3	104.5	1.13	34.3	37.3	0.92	1.23
B	92.3	91.3	1.01	24.8	26.9	0.92	1.10
C	122.6	68.7	1.79	20.5	19.5	1.05	1.71
D	19.0	10.9	1.74	5.95	5.48	1.09	1.60
E	54.5	40.1	1.36	13.7	13.8	0.98	1.39
22A	10.6	8.6	1.23	2.94	2.65	1.11	1.11
21C	14.4	7.3	1.97	2.84	3.12	0.91	2.16
F 3A – D	62.1	38.1	1.63	11.8	11.2	1.06	1.54
3A	11.2	6.2	1.80	3.12	2.18	1.43	1.26
3B	11.6	8.6	1.37	3.31	3.22	1.03	1.33
3C	15.7	6.5	2.42	3.88	2.65	1.46	1.66
G 3A – C	25.2	20.0	1.26	6.33	6.30	1.00	1.26
3A	10.3	8.2	1.26	2.67	2.54	1.05	1.20
3B	4.47	3.03	1.47	0.93	1.27	0.74	1.99
3C	10.9	7.4	1.47	2.90	2.90	1.00	1.47

[1] Same chromosomes as in Table I.
[2] See Materials and Methods for measurement of area and length.
[3] Ratio of areas corrected to equal lengths for diploid and haploid. See text.

chromosomes are superimposed, they will appear to occupy about the same area as their haploid homolog, but in this case the path length through them will be about twice as long. Intermediate situations are to be expected.

The chromosomes also, as noted elsewhere [4], are subject to being stretched during the preparative techniques. Differences between the area of diploid and haploid in the image plane that are the result of differential stretching can be eliminated by comparing only those pairs for which the two lengths are nearly equal, or by correcting for differences in length where found. For this material in which neither chromosome of a pair is more than fifty per cent longer than the other (Table III), a simple correction can be used. Stretching takes place mostly in the interband spaces; the bands seem to retain their size and shape, but are farther apart the greater the stretch, somewhat like the folds in an accordion bellows. As a first approximation, therefore, we can assume that the area is proportional to the length where the length alone is varied. The ratio of the observed areas divided by the

ratio of the observed lengths is the corrected ratio of areas. It is now proportional to the ratio of the average widths of the chromosomes and therefore inversely related to ratio of average thicknesses of the chromosomes.

We observe that the estimated ratios of the widths do fall within the expected extremes of one and two, within the limits of error of the measurements (Table III). Furthermore, the long chromosome regions that include one or more spirals are in the middle of the range; the highest values occur in the very short sections where only a very small portion of a twist is being examined.

More detailed comparison between Tables I and III shows that there is no correlation between the estimated ratio of widths (Table III) and the sign or the magnitude of the deviation from a 2:1 ratio of total extinction (Table I). For example, the diploids in B and E_1 are relatively thick (roughly equal in width to their respective haploids) and both negative and positive deviations are found. Conversely, in C and in E_2 the diploids are only one chromosome thick (width about twice that of their respective haploids) and the deviations are very small in both cases, positive in E_2, negative in C. Within the limits of error of the method then, the extinction must be proportional to the thickness of absorbing material within the range represented by one and two chromosomes.

DISCUSSION

Methodology.—It is worth noting that the conclusions presented here are not affected by many of the errors that may be encountered in the method. By comparing two chromosomes from the same nucleus, many biological and preparative variables are eliminated. By comparing them in a single field of the microscope, the same optical conditions are assured for the imaging of both objects. Finally, the use of only one photographic plate for a comparison eliminates a third source of unwanted variability.

The absolute values of total extinction may be subject to greater error than the relative values we have used. If so, then that error must be proportional to the total extinction. For example, if the measured extinction were too high because of non-specific light loss within the specimen, then that loss is twice as great in diploid as in haploid chromosomes. If it were the same in both, the real ratio between the two would be greater than 2:1 (would in fact be $(2-x):(1-x)$ where x is the assumed constant non-specific light loss). It seems quite improbable that such a loss would be of just the correct magnitude in each of so varied a collection of chromosome segments as to correct the measured ratio to 2:1. The same argument applies, of course, to any

other possible source of error. We may safely conclude, therefore, that the observed ratio is the real ratio of total extinctions of diploid and of haploid chromosomes in the same nucleus. It must also represent the ratio between the total masses of absorbing material in the two chromosomes as well.

Lambert's law.—We have used the term "Lambert's law" rather loosely to indicate proportionality between extinction and the number of chromosome thicknesses in the light beam. Properly speaking, the classical law relates extinction to optical path length in a space in which the chromophores exist in uniform concentration [20, 22]. This is certainly not the case in the chromosomes. Structural organization is visible within bands at the microscopic level, and sub-microscopic particles have been demonstrated with the electron microscope [3, 6, 24, 26]. If the chromophores in our material are grouped in structural packages such as these, then it becomes very difficult experimentally to draw the line between concentration and thickness in the photometric sense. On the one hand, we may in effect be counting rather large uniform packets, in all of which chromophore concentration must be considered constant, and we are really talking about Lambert's law. On the other hand, concentration gradients may be present between the centers of the structural elements of the chromosome. In that case, our data should be interpreted in terms of Beer's law as well. The simplest relation that would fit our data would then be the classical combination of both laws, i.e., that extinction is proportional to the product of concentration by path length. The experimental consequences of non-conformance with the Beer-Lambert law on the basis of this model would be the same as those we would look for if only Lambert's law were in question. So far as the data go, they are consistent with the idea behind both laws, that the absorbing units absorb light independently of each other. The practical significance of this finding is that the method is quantitative over a two-fold range in extinction and is valid for most, if not all of the conditions likely to be found in salivary gland chromosomes in pupating larvae of this species.

Pairing and the growth of chromosomes.—The biological significance of the data here presented lies in the finding that somatic pairing does not alter the total amount of absorbing material in the homologs. Since the nucleic acids and the proteins are responsible for most of the ultraviolet absorption, the diploid and haploid homologs must retain equal amounts of each of these groups of substances per chromosome. This discussion, of course, is restricted to materials which remain after acetic acid fixation.

This identity of material per chromosome must be interpreted in terms of the relation of the mechanisms by which homologs become associated

(pairing) and those which are responsible for replication (growth). It is well known [2, 23] that the pairing of the chromosomes in the salivary gland nuclei takes place before growth of the polytene chromosomes. The incompletely paired chromosomes cannot accomplish the association at later stages, and remain apart during the subsequent development [2, 27]. It follows, therefore, that the initial association of the chromosomes, even if it operated by some mechanism complementary to that of replication, would involve percentage-wise very little of the actual material of the chromosome as measured. Our data do not give therefore direct information about the primary mechanism of association in relation to growth. What they do provide is information concerning the relation of the geometry of the chromosomes to the final stage of growth.

From the fact that the haploid and the diploid homologs have the same amount of material, it may be inferred that the elementary units have multiplied to the same extent. Since, however, the surface volume relationships of the haploid and diploid homologs are not the same, the inference would also appear to be justified that those elements at the interior of the chromosome have access to materials for replication to the same extent as the ones more peripherally situated, e.g., after the model of Watson and Crick [13, 33]. The *in vivo* and electron microscope observations of salivary gland chromosomes could very well provide a mechanism (swelling and shrinkage of the chromosomes within the nucleus) for the distribution of intranuclear materials. Thus the units within the diploid homologs would be at no disadvantage as compared with those in the more easily accessible haploid. It is evident that further comparisons of the present kind under varied conditions of chromosome growth should provide more detailed information about this process.

SUMMARY

1. Measurements of the total amount of ultraviolet (chiefly 257 and 275 mμ) absorbing material in the salivary gland chromosomes of triploid *Drosophila melanogaster* have been made, using the photographic method for microspectrophotometry.

2. The total extinction of a paired diploid chromosome in a triploid nucleus is found to be twice that of the haploid homolog with which it has failed to pair. The deviations in measurements of parts of sixteen pairs of chromosomes in seven nuclei were found to be within the errors of the method.

3. Deviations were random with respect to the relative thickness of the diploid and haploid homologs. Lambert's law is, therefore, applicable to this material over a range of thicknesses of at least two to one.

4. The state of being paired is shown not to have an effect on the amount of material in fully grown salivary gland chomosomes.

The Köhler ultraviolet light source was constructed in the shop of the Institute for Cell Research and Genetics of the Karolinska Institutet, Stockholm, Sweden under the direction of Professor T. Caspersson. It is a pleasure to thank him for his generous help in this program of inter-laboratory cooperation.

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A SIMPLE ASSAY PROCEDURE FOR MATERIALS AND CONDITIONS IN TISSUE CULTURE

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In general, growth measurements have been used to determine tissue culture responses to media and culture conditions. The procedures include measurements of areal increases of explants [7], mitotic coefficients [7], net increases in cells [11] or their nuclei [10], increases in desoxyribonucleic acid [1], or increases in protein [4]. Parker [8] has adopted as a criterion the survival time of the last living cell from an explant.

Objections may be raised to growth as the chief measure in tissue culture. Growth is not necessarily the most characteristic physiological activity of tissue cells. The tissue cells are removed from the normal habitat and presumably stimulated to undergo drastic changes in metabolism. Thus is limited an important application of tissue culture, namely, the clarification of selected processes *in vivo*. Willmer [12] has pointed out the need for a definite, systematic study of the conditions necessary for cell survival.

The present paper describes a procedure for the simple, rapid assay of culture conditions and biologically active agents. The criterion for a positive response is not growth, but the establishment of healthy cells on the glass surface of a culture vessel within 16 hours. This method utilizes ordinary materials found in any laboratory, it is readily applicable to mass screening of chemical compounds, and it yields semi-quantitative results. Since the response may not depend upon drastic physiological changes (*i.e.*, the stimulation of the growth mechanism) it is possible that the conditions optimum for the establishment of the healthy cell will be those optimum for the normal activity of the cell. These latter may be differentiation, supporting metabolism, various physiological functions, or indeed, growth.

METHODS AND MATERIALS

General procedure.—The inoculum used was a cell suspension prepared from 10-day chick embryos. The procedure was similar to that of Dulbecco [2]. Blood and other fluids were washed from three 10-day chick embryos with Ca-free balanced salt

solution. The skin was removed from the back and the legs and washed in a small quantity of the salt solution. The fragments were placed in a 16 × 150 mm Pyrex test tube and mixed with 6 ml of trypsin solution. The tube was incubated for 20 minutes at 37°C with occasional vigorous swirling. A suspension of single cells and small clusters was collected in a 15 ml Pyrex centrifuge tube by filtering through a tuft of glass wool. The cells were centrifuged at 1000 rpm for 5 minutes. The supernate was removed and the residual drop at the lip of the test tube was absorbed with a gauze-wrapped cotton plug. The cells were resuspended in 5 ml of Ca-free salt solution and centrifugation was repeated. The cells were then resuspended in an appropriate amount of balanced salt solution. The cell concentration of the inoculum may be controlled by appropriate dilution after making a whole cell count exclusive of erythrocytes in a hemocytometer.

Solutions and media were mixed in desired proportions in test tubes. The cell inoculum was added and the final, well-mixed test suspensions were distributed to replicate culture tubes. The culture tubes used were prepared from standard wall Pyrex glass tubing 12 mm O.D. No. 00 rubber stoppers fitted very well. A flat portion, 10 × 14 mm, was molded on the side near the distal end of the tube, which was sealed off. There was a 3 mm indentation made immediately above the flat portion in order to confine the medium. The total length of the tubes was about 8 cm. A suitable volume of fluid for the tubes was 0.3 ml.

At the end of 16 hours the floor of the culture tubes was examined microscopically for the test response of the cells. Some typical responses are shown in Fig. 1. A negative response consists of the absence of spindle-shaped cells or fibroblasts on the floor of the culture tube. The cells may adhere more or less to the glass surface, but they are rounded up. In view of the enormous potency of embryo extract, incomplete washing of the initial cell inoculum or the use of heavy cell inocula possibly can lead to a few isolated spindles. Nevertheless, such a contingency would in no way invalidate results. A positive response consists of the presence of fibroblasts scattered throughout the tube in their characteristic spindle shape. Intermediate responses can result from gradation of factors. Thus the presence of a maximum number of spindles is indicated by a + + + response; less than maximum spindles, + +; the presence of scattered spindles, +; the presence of very few isolated spindles, ±; and no spindles, –. Of course, the density of cell distribution will depend upon initial cell concentration. However, by standardizing the cell concentration and by use of controls, this factor is of no concern. The culture flasks in these studies contained 150,000 to 300,000 cells/ml. Another aspect of response is deterioration of spindles to produce granular vacuolated cells of poorly defined form. This may occur in the presence of a poison, inhibitor, or certain unfavorable conditions.

By the careful preparation of a suitable cell suspension in which external materials are carried over in minimal amounts the test is reproducible and can be considered semi-quantitative in nature.

Balanced salt solution.—Earle's solution [3] was modified to contain 0.02*M* phosphate buffer rather than the usual bicarbonate buffer. This modification conveniently stabilizes the pH and results were the same in the assay as those obtained using bicarbonate buffered solutions. It was necessary to reduce the calcium content of the present salt solution to prevent the precipitation of calcium phosphate. The balanced salt solution had the following composition: NaCl, 6.88 g; KCl, 0.4 g;

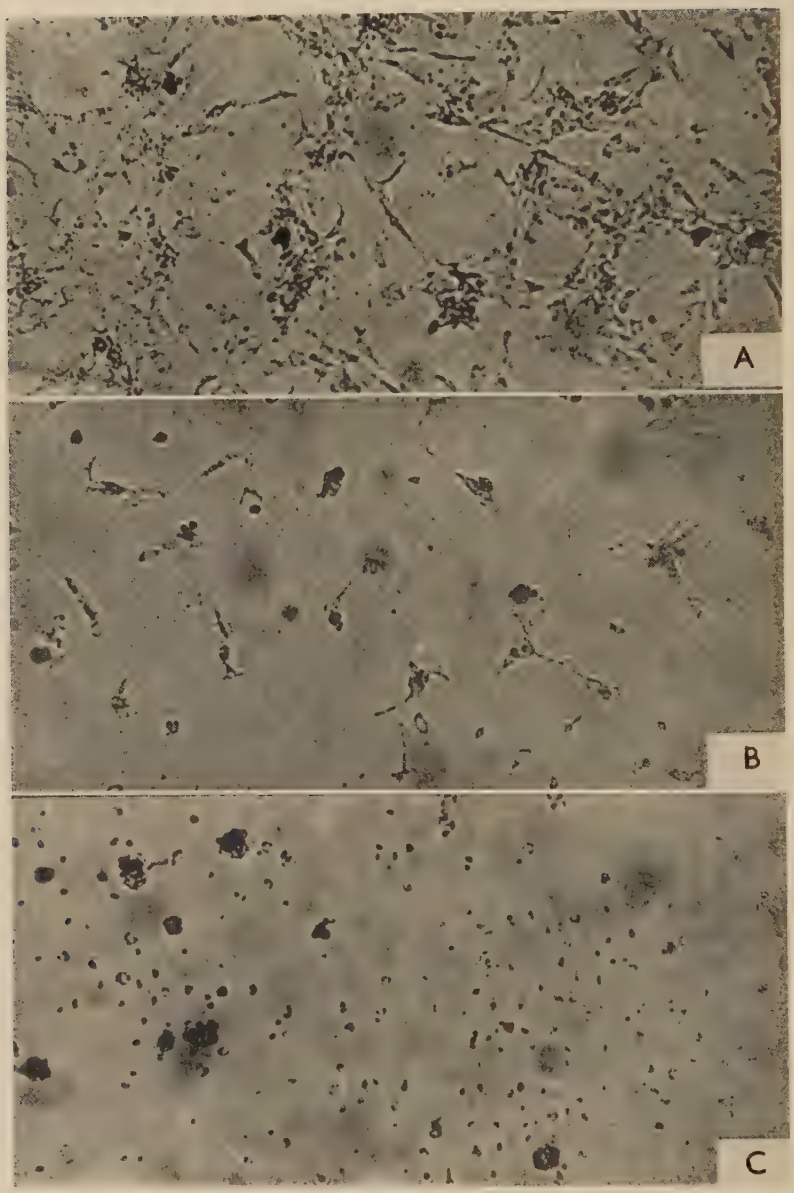

Fig. 1. Typical appearance of chick embryo cells *in vitro*. Magnification × 100, unstained.
A. + + + Response. Maximum number of spindles.
B. + Response. Note scattered spindles and some rounded cells.
C. − Response. Note all cells are rounded and no spindle cells are present.
(4) Osgood

$CaCl_2$, 0.1 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; $NaH_2PO_4 \cdot H_2O$, 0.55 g; Na_2HPO_4, 2.28 g; glucose, 1.0 g; glass distilled water to 1000 ml. The $CaCl_2$ was added as a 4 per cent solution to a cold solution of the other constituents. If necessary, the pH of this solution is adjusted to 7.4. Parshley and Simms [9] used a similar salt solution which contained no calcium or bicarbonate but did include aspartic acid and increased phosphate.

Embryo extract. —Ten-day chick embryos were washed with a small amount of balanced salt solution and forced through the orifice of a 20 ml Yale 20Y hypodermic syringe into an Erlenmeyer flask. For each embryo 2 ml of balanced salt solution was added. The flask was stored in the cold for one hour with occasional agitation. The mince was transferred to centrifuge tubes and centrifuged at 4000 rpm for 10 minutes. The supernate was distributed to heavy wall centrifuge tubes fitted with rubber stoppers and frozen. Immediately before use, the extract was thawed and centrifuged 5 minutes at 4000 rpm. This embryo extract, which was prepared fresh each week, appeared to be as potent as that resulting from overnight extraction and yielded a more consistent and usually a more active product than extracts resulting from immediate centrifugation.

Trypsin solution.—Difco-Bacto trypsin (1:250) was dissolved in Ca-free salt solution by warming to 37°C for 30 minutes with occasional agitation. The solution was sterilized by filtration through a Selas 03 filter and was distributed in 16 × 150 mm Pyrex test tubes stoppered with No. 1 rubber stoppers and frozen until used.

Dialyzation procedure.—The dialyzate was prepared by dialyzing the material in a Visking casing against an equal volume of salt solution. This procedure was carried out for 24 hours with continual shaking at 5°C. At the end of this time the residue was removed from the dialyzing solution and again dialyzed against 33 volumes of balanced salt solution under the same conditions previously described. This procedure was repeated to obtain the final residue which might be considered to contain only 0.05 per cent of the original dialyzable material.

Variation of pH.—In the studies of the effect of pH on the cells, the cell suspension and balanced salt solution contained no buffer. Embryo juice was used instead of embryo extract. The pH was controlled by using a 0.02*M* phosphate buffer. Various proportions of $NaH_2PO_4 \cdot H_2O$ and Na_2HPO_4 were added to attain the desired pH. The pH of each mixture was determined with a pH meter before incubation.

RESULTS AND DISCUSSION

The response of chick embryo cells to various culture media is shown in Table I. These results are in accord with the work of other investigators. It should be noted that the balanced salt solution always produced a negative response. Further, the embryo extract prepared in the manner described above proved exceedingly active since solutions as low as 1 per cent embryo extract have been found to be decidedly beneficial. The response to the samples of horse serum available gave an unexpectedly inferior result. However, homologous chick serum allowed very good spindle formation whether used in the presence of embryo extract or alone. It may be possible that the washed single cells freshly prepared from chick embryo skin were partic-

TABLE I

Response of chick embryo cells to various culture media.[1]

EE	HS	Response[2]	EE	CS	BS	DEE	DCS	EED	CSD	Response[2]
%	%		%	%	%	%	%	%	%	
0		−		5 – 40						+ + to + + +
1		+ to + + [3]	20	20						+ + +
2		+ to + + [3]			5 – 30					+ +
5		+ + to + + + [3]	20		20					+
15		+ + +				5 – 25				+ + +
20		+ + +						50		−
40		+ + +				25		50		+ + +
60		+ + +					5 – 40			+ +
	5 – 40	+							50	−
20	20	+					5		10	+ +

[1] EE = Embryo Extract
HS = Horse Serum
CS = Chick Serum
BS = Bovine Serum, Armour and Co.
DEE = Dialyzed Embryo Extract
DCS = Dialyzed Chick Serum
EED = Embryo Extract Dialyzate
CSD = Chick Serum Dialyzate
All media tested were made up to 100 per cent with balanced salt solution.

[2] Results: − no spindles; + scattered spindles; + + less than maximum spindles; + + + maximum number of spindles.

[3] Some variation between samples.

ularly sensitive to the heterologous horse serum. A similar series of experiments were carried out using tyrode solution [7] in place of the presently described balanced salt solution. The results obtained were closely similar to those reported in combinations of embryo extract, chick serum, horse serum, or balanced salt solution alone. It should be noted that dialyzed embryo extract and dialyzed chick serum yield a positive response, but the dialyzates of these materials yield a negative response.

On repeated trials a medium of approximately pH 7.4 promoted a consistently healthy appearance of the cells. This maximum response was attained over the range of 7.2 to 7.7. No spindles appeared at pH 6.0, while inferior response was recorded up to pH 6.8. At low levels of embryo juice (2 per cent) the inferior response at pH's up to 6.8 were intensified.

In order to observe the effect of relative tonicity on the cells, the concentration of sodium chloride in the balanced salt solution was altered over a range calculated to produce a relative tonicity of 0.54 to 3.0. The relative tonicity of 20 per cent embryo extract in balanced salt solution was ascribed the value of 1.0. The maximum response was recorded at the values 0.85 to 1.1, while

TABLE II

Response of chick embryo cells to various poisons, antibiotics and antagonists.[1]

Additive	Concentration	Response[2]	Additive	Concentration	Response[2]
NaF	0.25 m*M*	+ + +	S	2 – 2000 γ/ml	+ + +
,,	1.0 ,,	+ + +	,,	5000 ,,	+ + +[3]
,,	5.0 ,,	±	DIS	2 – 2000 γ/ml	+ + +
,,	10.0 ,,	–	,,	5000 ,,	+ +
NaN_3	0.5 ,,	+ + +	CA	5 – 50 m*M*	+ + +
,,	1.0 ,,	+ + +	GA	5 – 50 ,,	+ + +
,,	2.0 ,,	+ +	GA	5 – 50 ,,	+ +
,,	5.0 ,,	±	MS	50 – 100 ,,	+ +
,,	10.0 ,,	–	MS	10 ,,	+ + +
MA	2.0 ,,	+ + +	,,	50 ,,	+ +
,,	5.0 ,,	+ + +	,,	100 ,,	+ +
,,	10.0 ,,	+ + +	PR	1 – 200 γ/ml	+ + +
,,	20.0 ,,	+ +	,,	500 ,,	+ +
NaP	1.2 – 1200 γ/ml	+ + +	,,	1000 ,,	+ +
,,	3000 ,,	+ +	,,	2000 ,,	+

[1] NaF = Sodium fluoride
NaN_3 = Sodium azide
MA = Malonic acid
NaP = Sodium penicillin G
S = Streptomycin sulfate
DIS = Dihydrostreptomycin sulfate
CA = Cysteic acid
GA = L-Glutamic acid
MS = DL-Methionine sulfoxide
PR = Phenol red
Basal medium consisted of 15 per cent embryo extract and the solutions tested were made up to 100 per cent with balanced salt solution.

[2] Results: – no spindles; ± very few spindles; + scattered spindles; + + less than maximum spindles; + + + maximum number of spindles.

[3] Slightly inferior appearance.

a somewhat reduced quantity of spindles with some deterioration appeared at values 0.69 and 1.2–1.5, negative responses were recorded at values 0.54 and 2.0.

The response of the chick embryo cells to various poisons, antibiotics, and antagonists is shown in Table II. It is interesting to note that the presence of sodium fluoride and sodium azide, which presumably interfere with the anaerobic glycolysis of the cell, leads to the distinct destruction of the cells and yields a negative response. On the other hand, malonic acid, which interferes with the aerobic tricarboxylic cycle, had relatively little effect on the appearance of healthy spindle cells. The antibiotics, sodium penicillin G, streptomycin sulfate, and dihydrostreptomycin sulfate, were tolerated at all practical levels. The somewhat surprising response of the chick embryo cells

TABLE III

The effect of added cobalt and L-histidine to chick embryo cells *in vitro*.[1]

Cobalt added	Histidine added	Molar ratio Hist./Co	Response[2]
m*M*	m*M*		
0.035	–	–	+ + +
0.18	–	–	+ +[3]
0.35	–	–	±[4]
0.35	0.021	0.06	±
0.35	0.21	0.6	±
0.35	0.88	2.5	+ +
0.35	4.4	12.5	+ + +
0.35	8.8	25.0	+[5]
–	8.8	–	+ + +

[1] Basal medium consisted of 15 per cent embryo extract and the solutions tested were made up to 100 per cent with balanced salt solution.

[2] Results: – no spindles; ± very few spindles; + scattered spindles; + + less than maximum spindles; + + + maximum number of spindles.

[3] Some deterioration.

[4] Heavy debris obscures results.

[5] Much deterioration.

to amino acid analogues is worthy of note. When cysteic acid, an analogue of aspartic acid, was added in ranges from 5 to 50 m*M* it apparently had no effect on the formation of healthy appearing cells. Methionine sulfoxide, an analogue of glutamic acid, did have some effect on the number of spindles present. When combinations of methionine sulfoxide and glutamic acid were tested, glutamic acid did not overcome the slight inhibiting effect of methionine sulfoxide. The tolerance to phenol red is in accord with results previously reported [7].

Ethylenediaminetetraacetic acid (EDTA) was found to completely suppress spindle formation at 2.0 m*M* concentrations, though 0.1 m*M* concentration permitted the maximum response. The effect of 2.0 m*M* EDTA was overcome by the presence of 4.0 m*M* concentration of $MgSO_4$.

Morgan *et al.* [5, 6] reported growth inhibition when cobalt was added to a synthetic medium or to a plasma-embryo extract medium. Further, this inhibition could be overcome by the addition of histidine. The survival time of explants was reduced by two-thirds in a synthetic medium containing 10 μg of cobalt/ml. By using a molar ratio of 25:1 (histidine:cobalt) he was able to prevent the inhibitory effect of cobalt. This was particularly interesting since histidine was one of the few amino acids studied which did not inhibit

TABLE IV

The effect of trypsin, soybean inhibitor and serum on chick embryo cells *in vitro*.[1]

Trypsin added γ/ml	Response[2]	Trypsin added γ/ml	Additive	Response[2]
1	+ + +	10	20 % CS	+ +
5	+ + [3]	0	300 γ/ml SI	+ + +
10	± [4]	1	300 „ „	+ + +
50	±	5	300 „ „	+ + +
100	–	10	300 „ „	+ +
500	–	50	300 „ „	+ +
10 + 5 % BS	+ +	100	300 „ „	±
100 + 5 % BS	+	500	300 „ „	–

[1] BS = Bovine Serum; CS = Chick Serum; SI = Soybean Inhibitor. Basal medium consisted of 15 per cent embryo extract and the solutions tested were made up to 100 per cent with balanced salt solution.

[2] Results: – no spindles; ± very few spindles; + scattered spindles; + + less than maximum spindles; + + + maximum number of spindles.

[3] Some deterioration.

[4] Much deterioration.

the outgrowth of an explant in a plasma-embryo extract medium. In the present studies similar results were obtained (see Table III) but a lower molar ratio of added histidine was adequate to permit the normal response. Furthermore, the inhibition again appeared at higher concentrations of histidine in the presence of cobalt.

The effect of trypsin on chick embryo cells with and without the addition of soybean inhibitor and serum is shown in Table IV. Difco-Bacto trypsin (1:250) was definitely deleterious at concentrations as low as 5 μg/ml. Soybean inhibitor and serum (bovine and chick) can counteract this effect when used in appropriate concentrations. On the basis of simple dilution the procedure described here may be calculated to eliminate the trypsin in the initial cell inoculum to levels well below noticeable toxicity. This test has been repeatedly used in this laboratory in studying the response of chick embryo cells to various biologically active agents and no effect ascribable to residual trypsin has been observed to date.

SUMMARY

A simple, rapid assay procedure for conditions and materials in tissue culture is presented. The test involves the use of washed chick embryo cell suspensions as the inoculum. Under favorable conditions, healthy appearing

spindle cells become established overnight on the surface of the culture vessels. Under sufficiently unfavorable conditions, no spindle cells will appear. Intermediate responses occur with gradation of factors rendering the test semi-quantitative in nature. The procedure may be carried out in any laboratory since no specialized materials and conditions are required. It is readily applicable to the mass screening of biologically active agents and it is surprisingly reproducible. The response of the chick embryo cells to various culture media, conditions, and additives are given. These results are in close agreement with those obtained through more tedious procedures by other investigators.

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STUDIES ON THE NUCLEUS OF GIANT CELLS OF *MICROCOCCUS CRYOPHILUS*[1]

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With available techniques, extension of our knowledge about the nature of the bacterial nucleus and its mode of division must come from studies on the larger bacteria. *Micrococcus cryophilus,* isolated and characterized by McLean, Sulzbacher, and Mudd [8], represents a suitable tool. DeLamater and Woodburn [5] have investigated the nuclear cytology of this organism and presented evidence for the mitotic nature of the nuclear divisional mechanism in vegetative cells.

As originally noted by McLean *et al.*, populations of *M. cryophilus* commonly show small percentages of abnormally large cells. These giant cells represent superior material for the study of chromosome morphology and the mode of nuclear division in this organism. It will be shown that such cells, in the course of their further development, may give rise by two cell divisions to a tetrad of four cells of normal size. Nuclear events during these two divisions, and certain persistent characteristics of the chromosomal complement, will be described in this paper. Some observations on cell division in this organism are included.

METHODS

Micrococcus cryophilus was grown on nutrient agar enriched with 0.2 per cent Bacto yeast extract. The temperature was maintained at 21° to 23°C. A 16-hour culture served as inoculum. Cytological preparations were made from cultures 4 to 6 hours old which contained large numbers of giant cells. Simultaneous staining for nuclei and cell wall was accomplished by the following procedure. Fixation was carried out on the agar in the vapors of 1 per cent osmium tetroxide for 3 minutes. Smears on cover slips were hydrolyzed in 1*N* HCl at 60°C for 6 to 8 minutes without air drying, and subsequently stained overnight in 0.25 per cent aqueous thionin containing 0.1 per cent thionyl chloride (nuclear stain). The cells were then treated with

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10 per cent tannic acid for 10 to 20 minutes, and stained in 0.25 per cent basic fuchsin for 3 to 5 minutes (wall stain). Freezing-dehydration was carried out overnight in absolute normal propyl alcohol chilled by solid CO_2, and was followed by a rinse in room temperature alcohol, clearing in xylol, and permanent mounting in Technicon mounting medium. The above procedure incorporates steps from the staining and dehydration technique of DeLamater [3] and the method of Cassel [2] for simultaneous staining of nuclei and cell wall of bacteria. The chromosomes, stained blue, are contrasted by the red coloration of the cell wall and septa.

Observations were made with a Bausch & Lomb research microscope equipped with a 1.4 N.A. achromatic condenser, a 1.4 N.A. 90 × apochromatic oil immersion objective, and 15 × compensating oculars. Monochromatic light of wave length 570 mμ was supplied by a 100 watt zirconium arc lamp and a Bausch & Lomb interference filter. Photographs were taken on Kodak Microfile film at a camera length of 20 inches. The undesirably high contrast of this film was reduced and resolution improved by utilizing light of wave length 460 mμ for photographic purposes. The final magnification on the prints was 4500 ×.

RESULTS

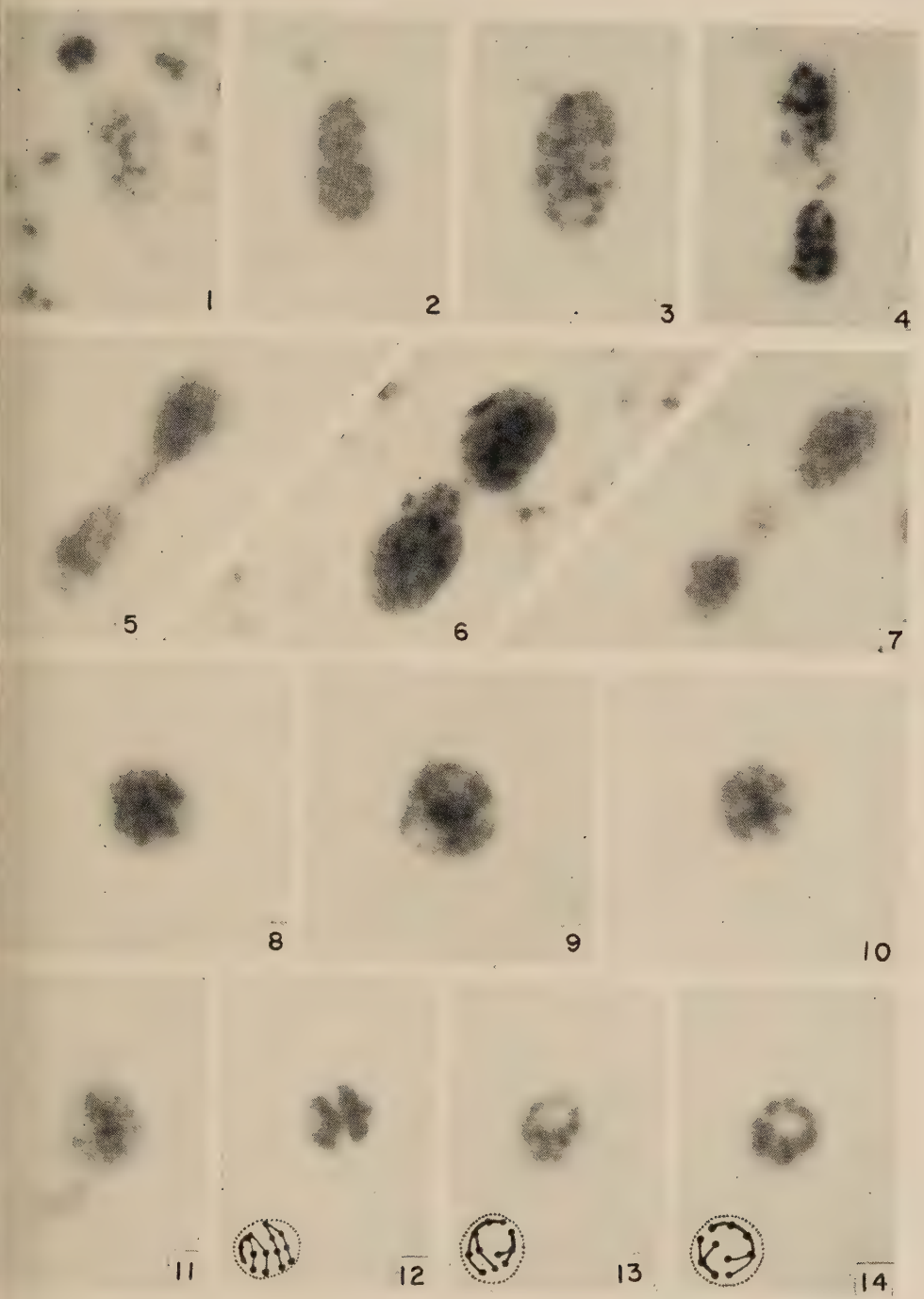
A characteristic feature of *M. cryophilus* is the formation at low frequencies of cells which are distinguished from the others by their extraordinarily large size. Such cells, here denoted giant cells, may measure 3.5μ in diameter. They invariably increase in number in old cultures, and inconsistently are found in large numbers in young cultures. Attempts to induce their formation consistently by varying the cultural conditions and the inoculum source were unsuccessful. The production of giant cells in large numbers is apparently dependent on a delicate balance of as yet undefined factors.

In slide cultures followed by phase microscopy, giant cells have been observed to give rise by two cell divisions to a tetrad of four cells of normal size. The two cells resulting from the first division are of intermediate size, and remain associated until the second division is completed. Tetrad groupings are especially common in cultures containing many giant cells.

Also correlated with a high incidence of giant cells, there appear significant numbers of peculiar cell forms, examples of which are shown in Figs. 1–7.

Micrococcus cryophilus. From 4–6 hour cultures grown on nutrient agar at 21° 23°C. Figs. 1 to 46: stained for nuclei and cell wall. Fig. 47: stained for cell wall by Hale's method. Figs. 1 to 7: formation of giant cells. Figs. 8 to 29, 31 to 34: first division of giant cells. Figs. 35 to 46: second division of giant cells. Figs. 30 and 47: haploid cells. Magnification × 4500.

Figs. 1–7. Configurations of unknown significance, but suggesting cellular and nuclear fusion.
Figs. 8–11. Giant cells containing filamentous chromatinic material.
Figs. 12 14. Chromosomes visualizable as two individual pairs of different lengths. "Synapsis" in late stages.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The three-unit structure of Fig. 7 is common. Since it has not been possible to follow the formation of giant cells in slide cultures, their origin is hypothetical. That they may arise through some form of cellular and nuclear fusion, of which Figs. 1 to 7 are stages, is suggestive.

Because of their size, the giant cells permit a more definitive analysis of nuclear structure than the smaller cells. The following description is confined to the pattern of nuclear behavior during the two divisions by which a giant cell gives rise to a tetrad of small cells. Figs. 8 through 46 show cells stained simultaneously for nuclei and cell wall in various stages of these two divisions. The first division is shown in Figs. 1–14, and the second division in Figs. 35 - 47.

The writer is in agreement with the contention of DeLamater and Woodburn [5] that the nucleus of *M. cryophilus* is constituted of chromosomes in the classic sense, which are distributed to daughter cells by a mitotic mechanism. The accompanying figures illustrate chromosomes of giant cell nuclei, which during cycles of contraction and elongation display morphological similarity to the chromosomes of higher cells. Emphasis is placed on the "first prophase", during which stage the chromosomes in the giant cells are more elongated than in the corresponding prophase of mitosis in the small cells, and more can be discerned about their structure (Figs. 8 to 24). It is also during this phase that certain peculiarities of the chromosomes, characteristic of this division, become apparent and distinguish it from the mitotic cycle in the small cells. The accompanying interpretive drawings were made during microscopic observation of the photographed cells. They are included to assist in following the description of events and to compensate to some extent for the loss of clarity inherent in the photographic process.

Visualization of the chromosomes as individual entities is not possible until they have reached the degree of contraction shown in Fig. 12. It can be discerned in Figs. 12 to 14 that each nucleus contains four strands of chromatic material, which are grouped into two pairs. The members of each pair are alike in "chromomeric" structure and length, implying that they are ho-

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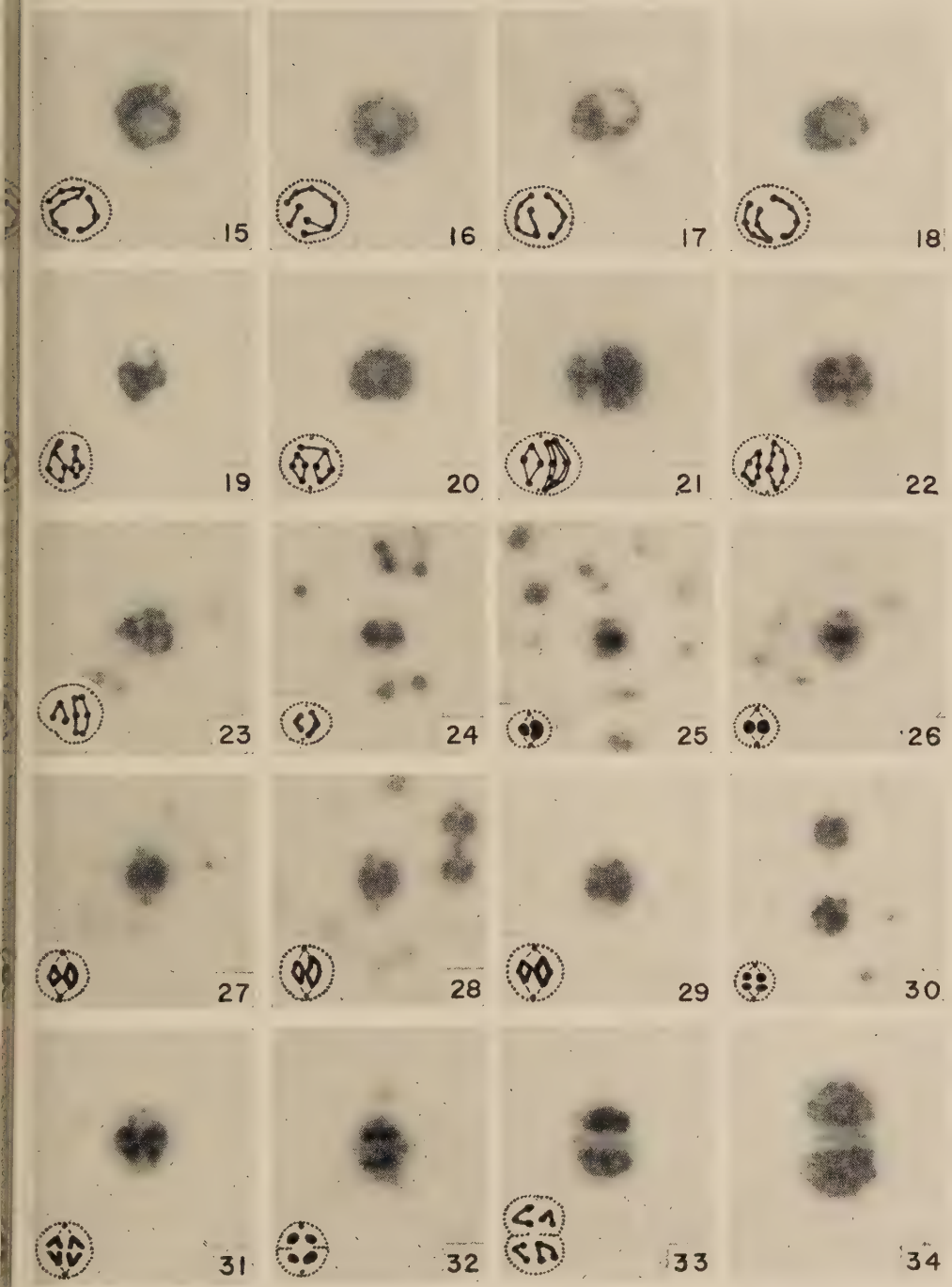
Figs. 15–18. Contracting chromosomes in peripheral position in nuclear area.
Figs. 19–22. Separation of homologues. Progressive contraction of two bivalents.
Figs. 23–24. Further contraction of bivalents. Contact of homologues terminal only.
Figs. 25–26. Lateral views of metaphase.
Figs. 27–29, 31. Anaphase separation of V-shaped chromosomes.
Fig. 30. Anaphase in haploid cells.
Figs. 32–33. First telophase. Septum formation. Beginning of re-elongation of V-shaped chromosomes (Fig. 33).
Fig. 34. Interphase. Individuality of chromosomes lost.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mologous chromosomes. They lie in longitudinal apposition with the association still incomplete at one end. Homologous regions of chromosomes are adjacent. These appearances are construed as late stages in the "synapsis" of homologous chromosomes in diploid cells. The implication follows that the minimum number of originally single chromosomes in the giant cell nuclei was four. The number of bivalents is two, which is taken as the haploid chromosome number. The possibility of the existence of unresolved smaller chromosomes is recognized. The figure is in agreement with the estimate of DeLamater [4] of the haploid chromosome number in this organism.

Distinction between the two bivalent chromosomes of the giant cells is possible on the basis of their relative lengths. The pattern of long and short chromosome is consistent and, as will be seen, is maintained throughout the two nuclear cycles. The large chromosome is approximately twice the length of the small chromosome, although the ratio may vary slightly depending on the relative rate of contraction of the chromosomes. The "chromomeres" appear to be distributed at about equal intervals along the chromosomes. Their apparent number decreases with contraction of the chromosome.

Figs. 15 to 18 show the chromosomes lying more or less peripherally in the nuclear area, which occupies a large part of the total cell volume. The apparent number of chromosomes is now two, although each thread is presumed to be a composite of two homologues. As contraction progresses further, the homologues become widely separated at points along their length (Figs. 19 and 20). In still later stages of contraction they appear to be associated terminally only (Figs. 21 and 22). Eventually contact at one end may be lost (Figs. 23 and 24), especially in the case of the short bivalent. These appearances of the chromosomes during the first prophase are reminiscent of loop formation and terminalization of chiasmata in meiotic chromosomes

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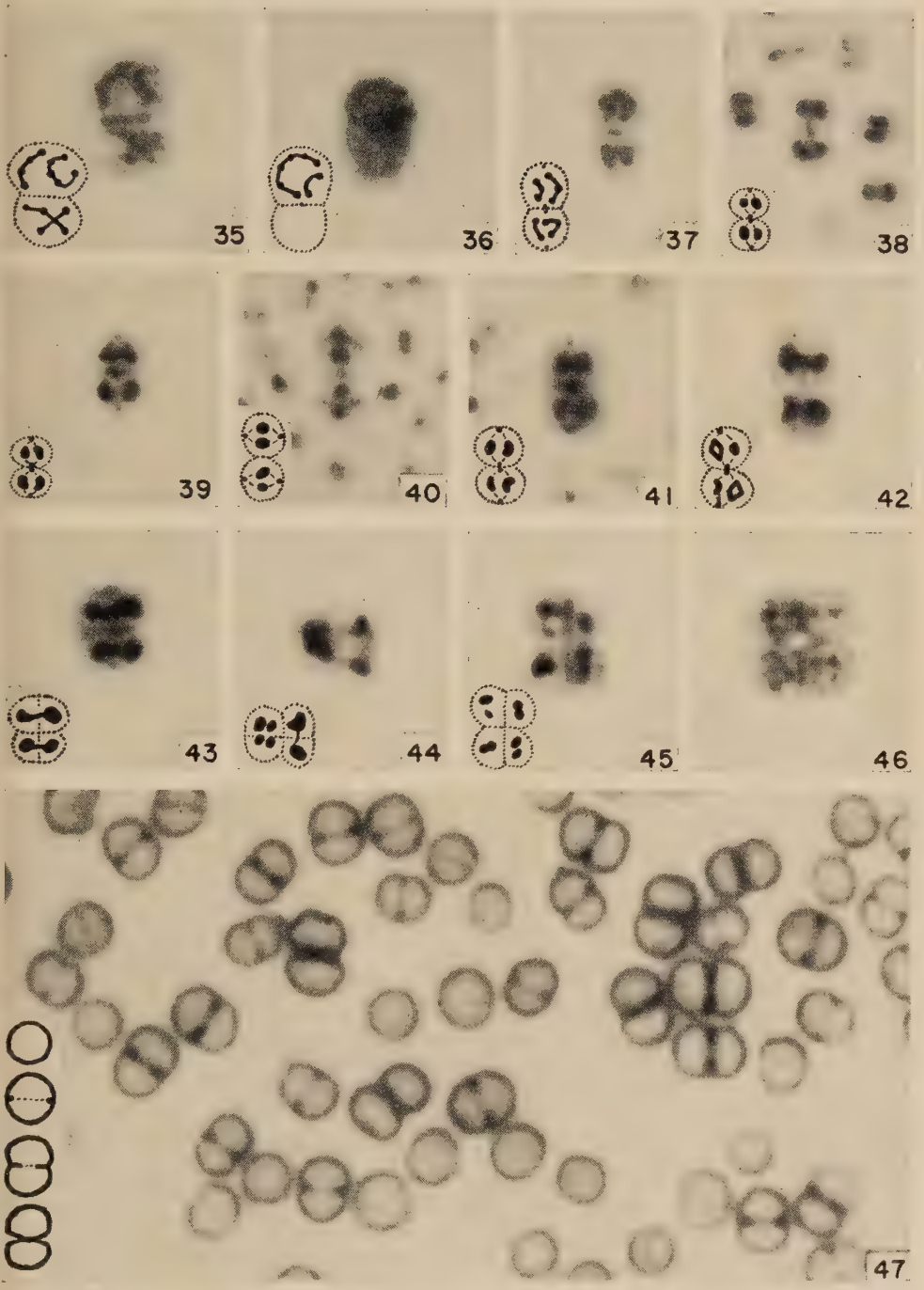
Figs. 35–37. Prophase of second division. Long and short chromosomes identifiable. Progressive contraction.
Figs. 38–40. Second metaphase. Two different orientations of axes of metaphase spindles.
Figs. 41–44. Second anaphase. Septum formation (Figs. 43 and 44).
Figs. 45 46. Second telophase. Tetrads of haploid cells. Two contracted chromosomes sometimes countable. Chromosomes re-elongated (Fig. 46).
Fig. 47. Actively growing haploid cells, stained for cell walls by Hale's method. Septum formation by ingrowth of annular ring, simultaneously with cell constriction. Actual separation of the protoplasm of the two sister cells occurs at a late stage just prior to cell division.

Note added at proofreading: The author regrets that owing to the fineness of the nuclear structure, loss of detail during the reproductive process was greater than anticipated. Reference to the diagramatic drawings must substitute for access to the original photomicrographs or preparations.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of higher forms. The long bivalent of Fig. 21 could be readily visualized microscopically as being four-stranded, although this detail was lost in the photographic process.

Figs. 25 to 29 and 31 to 33 show metaphase, anaphase, and telophase stages of the first division. Morphological individuality of the chromosomes is retained. The separating chromatids appear V-shaped at anaphase of both divisions, suggesting median or sub-median centromere positions. Two haploid cells in anaphase (Fig. 30) are included to show their smaller size. An interphase (Fig. 34) intervenes between the two divisions, in which the chromosomes as long tangled threads remain visible, although their individuality is obscured.

The second division (Figs. 35 to 46) can be followed in both products of the first division, which remain associated. The chromosomes are visualized in prophase as two individual threads, distinguishable from one another by their length (Figs. 35 to 37). Figs. 38 to 40 show two possible orientations of the axes of the two metaphase spindles. Tetrads of cells are the end products of the two divisions. Chromosome counts made on the small progeny of the giant cells during telophase of the second division (Figs. 44 and 45) indicate that their nuclei contain only two visualizable chromosomes. Equivalence of the two nuclear divisions described here to the meiotic process in higher forms is conceivable.

Septum formation in both the giant cells and small cells appears to take place by ingrowth of an annular ring from the cell wall (Figs. 32 to 34, 43 and 44). The nature of the cell plate-like structure recorded in the photographs of DeLamater and Woodburn [5] is at present not clear. When stained for cell walls by the tannic acid method or by the technique of Hale [6], *M. cryophilus*, regardless of size, appeared either unicellular or divided by a partially completed septum. "Haploid" cells stained by Hale's method are shown in Fig. 47.

Minute basophilic bodies, appearing opposite each other at the periphery of cells in certain stages (Figs. 20, 25 to 32, and 37 to 42), correspond to like structures described by DeLamater and Woodburn [5] and interpreted as centriolar. It is possible that when visualized they do not represent bare centrioles but include adsorbed dye or some additional stainable material. Their size varies in a cyclic fashion. They are largest at metaphase, becoming invisible during telophase, and reappearing again in the next prophase, at which time two may be visualized in various stages of migration. The existence of the remainder of an achromatic figure at metaphase is only barely suggested (Figs. 25, 26, 39 to 41).

DISCUSSION

Giant cells of *M. cryophilus* offer a technical advantage for the cytological study of intracellular structures which give the staining reactions of DNA. These structures seem to be equivalent to the chromosomes of higher forms. Morphologically, the chromosomes of *M. cryophilus* are threadlike structures which undergo a cycle of contraction and elongation during nuclear division. Some manner of longitudinal organization is suggested by their beaded appearance. Evidence that they are distributed to daughter cells by means of a mitotic mechanism has previously been presented by DeLamater and Woodburn [5]. During division of the giant cells the chromosomes give appearances which resemble meiotic chromosomes of higher forms. The haploid chromosome number has been placed at a minimum of two, a confirmation of the earlier estimate of DeLamater [4]. Morphologically, the complement consists of one long and one short chromosome. Although they cannot be followed as individuals through the interphase separating two divisions, evidence for the continuity of the chromosomes is provided by the reappearance in prophase of the two morphologically definable chromosomes. Apposition of homologous regions of chromosomes during what is considered synapsis in diploid cells indicates constancy of the longitudinal organization provided by chromomeres.

Studies described here have been confined to the giant cells of *M. cryophilus*. Size alone is obviously not a significant characteristic, since it may vary with a number of factors. However, certain observations suggest that the giant cells may have some special significance in the life cycle of the organism. Although it has not been possible to observe their formation in slide cultures, their origin in some form of cell fusion process is suggested by the peculiar cell forms of Figs. 1 to 7. Further study is needed to clarify the nature of this phenomenon. The doubled chromosome number in the giant cell nucleus indicates its origin by nuclear fusion or by some unknown process of diploidization.

Further significance is added to the presence of giant cells by a study of chromosomal behaviour during the two cell divisions by which a giant cell gives rise to a tetrad of four small cells. The postulation that the giant cells are diploid and that these two divisions are meiotic is based on the halving of chromosome number judged by counts made on the giant cells and the tetrad of small cells, apparent chromosome synapsis, and certain peculiarities in the chromosomes during prophase of the first division characteristic of diplotene chromosomes of higher forms. In general outline the formulated

meiotic process in *M. cryophilus* follows the classic pattern, although limitations imposed by size prevent a more detailed analysis of the mechanism at this time. It is not apparent whether there is any similarity to the reduction divisions in a diplococcus described by Lindegren [7].

The sequence formulated here is based on cell size, chromosome length, and stage in septum formation. Cell size and nuclear volume in stained preparations vary directly with chromosome length, which is least at metaphase. Admittedly the sequence has been derived from observations on dead cells, but its tentative presentation is felt justified in the absence of any means for making similar studies on living cells.

The use of photographs of single cells as illustrative examples is a technical necessity. It arises due to the rarity of an orientation of intracellular structures such that they lie entirely in a single shallow focal plane and appear in a single photograph. Overlying of such structures or their close proximity may prevent conclusive interpretation in a cell as small as the bacterial cell. The interpretations presented here are, therefore, based on appearances in the majority of cells examined, using selected cells for photographic documentation.

The contention of Bisset [1] that *M. cryophilus* is a predominantly multicellular organism has not been confirmed. Cocci from actively growing cultures (4 to 6 hours old), stained for cell walls by Hale's method [6], appear either unicellular or with a partially completed septum (Fig. 47). Four-celled cocci were never observed. At least a portion of the four-celled organisms described by Bisset may be accountable as equivalent to the configurations of Figs. 43 to 45. Such configurations are here construed as two sister cocci which, while still in close association, are undergoing division simultaneously. It is difficult to compare results, since Bisset did not supply technical details, such as temperature of incubation and culture medium. Both the 2-hour- and 24-hour-old cultures stained and illustrated by Bisset undoubtedly consist of old cells, since this is a slow-growing organism.

The use of simultaneous cell wall and nuclear stains of different colors in this study has made confusion of nuclear material with wall or membrane structures improbable. The plane of cell division, as indicated by the ingrowing septum, is at right angles to the axis of spindle elongation (Figs. 32 and 44), making it seem unlikely that "granules at the junction of cell wall and cross wall" [1] have been misinterpreted as centrioles. It should be pointed out that distinction between centrioles and primordial septa is difficult in photographs, but can readily be made during microscopical observation by variable focusing since the primordial septa are actually annular rings.

Predominant uninuclearity and morphological definability of the chromosomal complement give *M. cryophilus* some promise as a cytogenetic tool, although size is still a severely limiting factor. Further advantages may lie in the proposed operation of a cytologically detectable sexual process and of a seemingly classical mechanism for segregation of genetic factors with survival in association of all four products of meiosis, making tetrad analysis theoretically feasible. A cytological basis is provided for genetic continuity of the chromosomes, linkage, and crossing-over.

SUMMARY

The minimum haploid chromosome number of *Micrococcus cryophilus* is two. The chromosomes have been identified at two successive nuclear divisions on the basis ot their relative lengths. Giant cells of *M. cryophilus* are considered to be diploid and to arise by some form of cellular and nuclear fusion. In the course of two nuclear divisions, they give rise to four haploid cells.

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Experimental Cell Research, **9**, *232–240 (1955)*

ANIMALISATION DE L'ŒUF D'OURSIN PAR LES COLORANTS AZOÏQUES ET LES BLEUS D'ANILINE SULFONÉS

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Le développement embryonnaire de l'œuf d'oursin peut être modifié expérimentalement par des substances très variées. L'ion lithium exerce une action végétativisante [10]. Celle-ci se traduit notamment par une extension des régions entodermiques aux dépens des régions ectomésodermiques, entrainant ainsi l'inhibition plus ou moins marquée du développement de la touffe ciliée apicale.

D'autres substances, l'ion sulfocyanure par exemple, ont au contraire un effet animalisant, caractérisé par l'extension des régions ectodermiques aux dépens des régions entomésodermiques. En conséquence, la touffe ciliée apicale est ici très développée, tandis que l'archentéron est peu différencié ou absent.

Selon la conception de Runnström [18, 19], un système de deux gradients antagonistes, l'un de type animal, l'autre de type végétatif, régit l'équilibre entre les régions ecto et entomésodermiques. Les travaux de Lindahl [14] et de Gustafson [5] ont apporté une contribution importante à l'élucidation de la nature de ces gradients.

Nous décrivons dans ce travail, l'effet de certains colorants sur le développement embryonnaire de l'œuf d'oursin. Les colorants employés appartiennent à deux groupes de structures chimiques différentes, dérivés azoïques: bleu et rouge trypan, rouge Congo, et dérivés du triphénylméthane : bleu à l'eau et bleu de méthyle.

MATÉRIEL ET MÉTHODES

Ces expériences ont été faites avec les œufs de l'oursin *Paracentrotus lividus*. Les œufs lavés trois fois à l'eau de mer filtrée, sont fécondés par addition d'une goutte de suspension de spermatozoïdes, puis transférés dans des cristallisoirs contenant chacun 40 cc de solution de colorant dans l'eau de mer filtrée.

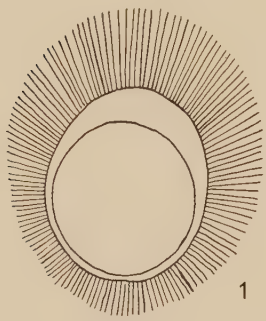
Les colorants utilisés : bleu et rouge trypan, rouge Congo, bleu à l'eau et bleu de méthyle, sont dissous au moment de l'emploi dans l'eau de mer filtrée. Une solution à 1/5.000 fraîchement préparée, est utilisée pour préparer les solutions plus diluées. Le bleu et le rouge trypan, le rouge Congo se dissolvent difficilement. Il se produit en général après quelques jours, un léger précipité de colorant. Le bleu à l'eau et le bleu de méthyle sont très solubles dans l'eau de mer. Les solutions diluées, à 1/50.000 notamment, se décolorent en quelques heures à la lumière, dans les cristallisoirs de culture. Les embryons sont laissés en permanence ou temporairement dans les solutions de colorant. Dans les cas de traitement temporaire, les embryons sont rassemblés par une centrifugation à faible vitesse, lavés trois fois, puis transférés dans l'eau de mer filtrée. Toutes les cultures sont faites à la température du laboratoire. Les dessins sont exécutés à la chambre claire à un fort grossissement.

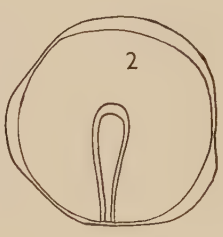
RÉSULTATS EXPÉRIMENTAUX

Bleu trypan. Dans les solutions à 1/5.000, la vitesse de segmentation est normale; les embryons ne dépassent pas le stade blastula. Ces blastulas, laissées dans la solution de colorant, n'éclosent que très rarement. A l'examen microscopique, les parois épaisses apparaissent légèrement colorées en rose violacé. Les cellules mésenchymateuses primaires, groupées au pôle végétatif, sont intensément colorées en lie de vin. De petites cellules rondes, colorées en rouge rubis, sont disséminées dans le blastocèle. De longs cils recouvrent toute la surface de la blastula. Ils sont incurvés en raison de l'exiguité de l'espace compris entre l'embryon et la membrane qui l'enveloppe. Très mobiles, ils communiquent à la blastula un lent mouvement de rotation. Quelques embryons se lysent, mais la plupart conservent cet aspect, parfois une semaine, sans se développer, ni éclore. La coloration générale s'intensifie progressivement et la mobilité ciliaire est conservée, mais la taille de ces embryons diminue sensiblement. Quelques blastulas parviennent à éclore dans les solutions de bleu trypan à 1/50.000. Leur épaississement ectodermique très accentué porte une touffe ciliée apicale élargie. La ciliature fait bientôt place à un champ cilié constitué de cils courts et mobiles recouvrant tout l'embryon. Des cellules mésenchymateuses primaires fortement colorées, certaines en rouge rubis, d'autres en lie de vin foncé, sont groupées au pôle végétatif. L'archentéron et les spicules sont absents. Après un séjour de 25 à 29 heures dans le colorant, suivi d'un transfer dans l'eau de mer, l'éclosion est la règle. Chez les blastulas écloses, les caractéristiques de l'ectoderme et de la ciliature sont analogues à celles que nous venons de décrire. Les cellules mésenchymateuses sont disséminées dans le blastocèle. On trouve également, dans ces cultures, un grand nombre de gastrulas avec un archentéron réduit à une ou deux vésicules. Les spicules triradiés, de petite taille, sont

en nombre variable, deux à cinq en général. Des cellules colorées en rouge s'accumulent au dessous de l'épaississement apical.

Les embryons éclosent directement dans les solutions à 1/10.000. Ils se libèrent très lentement par un orifice percé dans la membrane. Les blastulas hyperciliées (Fig. 1), sont fréquentes. Quelques unes ont des spicules très





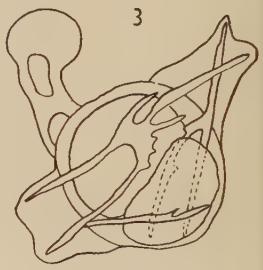


Fig. 1. Blastula hyperciliée (Bleu trypan 1/10.000).
Fig. 2. Embryon après 76 heures de séjour dans le bleu trypan à 1/10.000.
Fig. 3. Pluteus à lobe oral hyperdéveloppé (bleu trypan 1/50.000).

ramifiés, en position végétative. On remarque également des embryons pourvus d'une bande ciliée; l'archentéron est étroit et non segmenté (Fig. 2).

Les cultures permanentes, dans les solutions à 1/20.000, sont constituées essentiellement par des gastrulas. On y trouve en outre, quelques pluteus à lobe oral hyperdéveloppé. Ces derniers se trouvent en abondance dans les solutions à 1/50.000. Dès le stade gastrula, on observe un allongement de la région apicale, aboutissant ultérieurement à la formation d'un lobe oral. L'ectoderme de ce lobe est très épais et recouvert de longs cils mobiles formant une touffe large à son extrémité. Le corps de l'embryon, situé au dessous de ce lobe oral, forme une base élargie à contours irréguliers, crénelés. Dans cette base, immédiatemment au dessous du lobe oral, on trouve des spicules très grands et ramifiés. L'archentéron est différencié en trois vésicules. La bouche est présente. Ultérieurement les bras anaux se développent, rejetés latéralement (Fig. 3). Le lobe oral très allongé, se rétrécit progressivement à sa base, juste au dessus de la bouche (Fig. 4). Ce processus s'achève par la séparation complète du lobe oral, qui forme une petite sphère ectodermique ciliée libre (Fig. 5). Les embryons laissés 29 heures dans la solution de bleu trypan à 1/50.000, puis transférés dans l'eau de mer, se développent d'une façon analogue; leur viabilité est toutefois légèrement plus grande que dans les cultures précédentes. Dans les solutions plus diluées,

1/100.000 et 1/200.000, se développent également des pluteus à lobe oral hyperdéveloppé.

Rouge trypan. A la concentration 1/5.000, le rouge trypan ne ralentit pas la segmentation. L'éclosion n'est que légèrement retardée. La membrane est complètement dissoute et l'embryon se libère sans difficulté. Dans ces

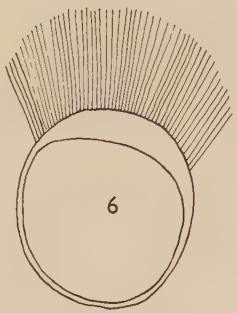


Fig. 4. Pluteus avec lobe oral en voie de séparation (bleu trypan 1/50.000).
Fig. 5. Sphère ectodermique ciliée formée par le lobe oral après sa séparation du corps de l'embryon (bleu trypan 1/50.000).
Fig. 6. Blastula (rouge trypan 1/5.000).

cultures, on trouve des blastulas avec un épaississement apical très marqué, recouvert d'une touffe ciliée élargie (Fig. 6). De jeunes gastrulas, avec un archentéron réduit sont également nombreuses. Leurs cellules mésenchymateuses allongées, s'étendent dans le blastocèle, entre l'archentéron et les parois de l'embryon. Ces jeunes gastrulas se transforment en embryons ovoïdes, munis d'un champ cilié animal ou d'une bande ciliée. L'archentéron de petite taille est différencié en deux, parfois trois vésicules. La bouche n'est pas formée. Les cellules mésenchymateuses sont rares. Des cellules rondes, colorées en rouge, sont disséminées dans le blastocèle. Les spicules sont absents. Ces embryons apparaissent colorés en rouge clair à l'examen direct.

Dans les solutions à 1/50.000 et à 1/100.000, on remarque dès la gastrulation, l'allongement de la région apicale. Chez les pluteus, le lobe oral est hyperdéveloppé. Ces embryons ont une structure analogue à celle des pluteus cultivés dans les solutions de bleu trypan à 1/50.000. Les formes ovoïdes, avec un archentéron réduit, présentes dans les solutions à 1/50.000, ont totalement disparu dans les solutions à 1/100.000.

Rouge Congo. Dans les solutions de rouge Congo à 1/50.000, la segmentation est normale. Les gastrulas à parois épaisses, fortement colorées en jaune restent dans leur membrane. L'épaississement ectodermique apical est très accentué. Ces embryons sont couverts de cils longs et mobiles. L'archen-

téron et les spicules sont normaux. Ces embryons, laissés dans la solution, n'éclosent généralement pas; leur taille diminue sensiblement. Quelques rares blastulas sphériques éclosent. Elles sont recouvertes d'un champ cilié uniforme, constitué de cils courts et mobiles. Les cellules mésenchymateuses colorées en rouge orangé sont dispersées dans le blastocèle. Les spicules manquent.

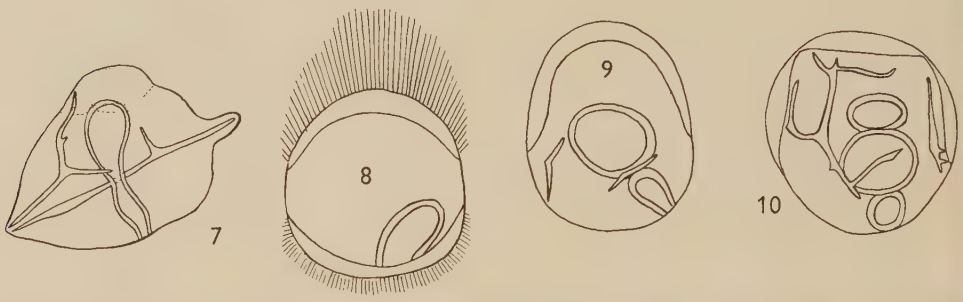


Fig. 7. Embryon laissé dans le rouge Congo, 1/50.000, pendant 27 heures puis cultivé dans l'eau de mer normale.
Fig. 8. Embryon après 79 heures de séjour dans le rouge Congo à 1/10.000.
Fig. 9. Embryon après 79 heures de séjour dans le rouge Congo à 1/20.000.
Fig. 10. Embryon après 79 heures de séjour dans le rouge Congo à 1/50.000.

Après un séjour limité dans la solution de colorant, l'éclosion s'effectue si toutefois, le temps de séjour n'a pas été trop prolongé. Après un séjour de 99 heures, peu d'embryons éclosent. Ils sont fortement colorés, de forme générale globuleuse. Leur archentéron est réduit à une petite vésicule. La bouche est absente. Après 27 heures de traitement, l'effet du colorant est encore notable: on rencontre des formes globuleuses avec champ cilié apical et archentéron différencié en deux ou trois vésicules, mais la bouche est absente. Les spicules sont en général présents. Des embryons du type représenté par la Fig. 7, sont également abondants. La diminution de la durée du traitement, entraine l'accroissement du nombre des pluteus à lobe oral hyperdéveloppé.

Dans les solutions à 1/10.000, les éclosions sont relativement fréquentes. On obtient des blastulas à champ cilié apical et des embryons ovoïdes, avec un archentéron réduit à une petite vésicule (Fig. 8).

Les embryons restent ovoïdes dans les solutions à 1/20.000 et à 1/50.000, mais l'archentéron est plus différencié (Figs. 9, 10). Les embryons laissés pendant 27 heures dans les solutions de colorant à 1/10.000 et à 1/20.000, donnent des pluteus à lobe oral hyperdéveloppé.

Bleu à l'eau. Le bleu à l'eau, à la concentration 1/5.000, apparaît sans action sur la segmentation. L'éclosion est, par contre, fortement inhibée. Les blastulas ont un épaississement ectodermique apical très marqué. Les parois sont colorées en bleu, assez faiblement d'ailleurs, tandis que les cellules mésenchymateuses, groupées au pôle végétatif, sont intensément colorées en bleu. Des cellules rondes, rouges, sont éparpillées dans le blastocèle. Des

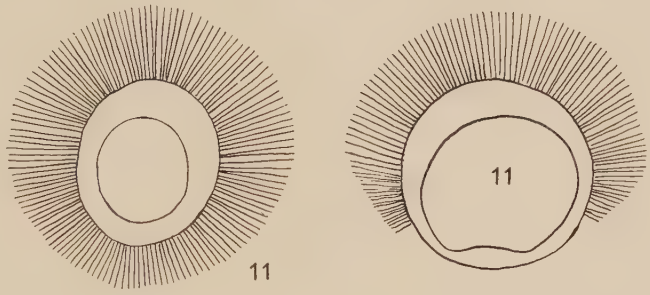


Fig. 11. Blastulas (bleu à l'eau 1/5.000).

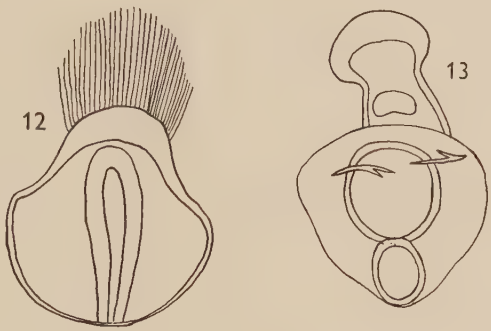


Fig. 12. Gastrula (bleu de méthyle 1/50.000).
Fig. 13. Embryon à lobe oral hyperdéveloppé (bleu de méthyle 1/50.000).

cils très mobiles recouvrent tout l'embryon. Celui-ci reste en général dans cet état sans éclore, pendant une semaine, tout en conservant sa mobilité. Fréquemment les parois se plissent et les contours de l'embryon deviennent très irréguliers. Quelques embryons éclosent : l'opération est très lente, l'embryon faisant issue progressivement par un orifice percé dans la membrane. Les blastulas écloses portent une touffe ciliée très large (Fig. 11).

Après un séjour de 27.30 heures dans la solution de bleu à l'eau à 1/5.000, les embryons reportés dans l'eau de mer, donnent des blastulas hyperciliées et des gastrulas avec une petite vésicule archentérique. Un petit nombre seulement d'embryons éclôt dans ces conditions. Ils se lysent d'ailleurs plus rapidement que les embryons laissés dans la solution de colorant.

Les embryons cultivés, de façon permanente, dans les solutions à 1/50.000

se différencient en pluteus à lobe oral hyperdéveloppé. Ces pluteus ressemblent à ceux observés dans les solutions de bleu trypan à 1/50.000.

Bleu de méthyle. Le bleu de méthyle est plus toxique que le bleu à l'eau. Dans les solutions à 1/5.000, la plupart des embryons sont bloqués au stade morula. Les rares embryons éclos sont des blastulas, à parois ectodermiques égales ou inégales, recouvertes de cils très allongés.

Les solutions à 1/10.000 sont moins toxiques. Quelques embryons s'y développent en formant des blastulas hyperciliées et des gastrulas ovoïdes, avec une petite vésicule archentérique. Ces embryons sont peu viables et se lysent rapidement.

Dans les solutions à 1/50.000, les gastrulas ont une plaque apicale accentuée, avec un léger élargissement de la touffe ciliée apicale. Toute cette région de la gastrula s'allonge, formant l'ébauche du futur lobe oral, très développé chez le pluteus (Fig. 12). Chez ces derniers, les bras anaux ne sont pas formés. Les spicules restent petits (Fig. 13).

DISCUSSION

Les colorants utilisés dans ces expériences, bien qu'appartenant à des séries chimiques distinctes exercent une action animalisante sur le développement de l'œuf de l'oursin *Paracentrotus lividus.*

La vitesse de segmentation reste normale avec les colorants de la série azoïque : bleu et rouge trypan, rouge Congo. Les colorants de la série du triphénylméthane sont plus toxiques. Dans les solutions à 1/5.000 de bleu de méthyle, les premières divisions sont normales, mais le développement dépasse rarement le stade morula.

Tous les colorants étudiés ralentissent le développement à partir du stade blastula, et l'éclosion est fortement retardée, parfois même supprimée, si la concentration en colorant est élevée ou la durée du traitement prolongée.

Ces colorants ont des propriétés fortement électronégatives dues à leurs groupements sulfoniques. Ils peuvent donc se combiner avec les groupes electropositifs des protéines, pour former des complexes. Les travaux de Efskind [3], de Schubert et Wiegandt [21], de Bennhold et Seybold [1] notamment, ont montré la formation de tels complexes, entre le rouge Congo, le bleu et le rouge trypan, et les protéines. Haurowitz et ses collaborateurs [6] ont particulièrement étudié les complexes formés par l'ovalbumine et la sérumalbumine avec le rouge Congo. Ils ont observé que les protéines dénaturées fixent davantage de molécules de colorant que la protéine native. Il est intéressant de rappeler ici que dans toutes nos expériences les cellules

mésenchymateuses primaires apparaissent toujours plus colorées que le reste de l'embryon. Ce phénomène est observé avec tous les colorants étudiés. S'agit-il d'une plus grande perméabilité de ces cellules ou d'une aptitude plus marquée à fixer le colorant ? Dans cette dernière éventualité, il pourrait y avoir là l'indication, soit d'une accumulation de protéines basiques dans ces cellules, soit d'une dénaturation des protéines intracellulaires, entrainant l'augmentation des groupements basiques libres susceptibles de fixer le colorant.

L'affinité de ces colorants pour les protéines joue sans doute un rôle important au cours de l'animalisation produite par ces substances. D'autres substances également capables d'animaliser l'embryon d'oursin, forment des complexes avec les protéines; c'est le cas des ions zinc et cadmium [13]. Mais ici l'ion métallique électropositif présente une affinité pour les groupes électronégatifs de la protéine. Les conditions de fixation des colorants et des ions apparaissent ainsi dissemblables, et l'on conçoit que ces substances puissent agir par des voies différentes.

Si l'on examine la structure chimique des colorants utilisés, on remarque que tous portent des groupes sulfoniques. Ceux-ci déterminent, nous l'avons vu, l'affinité pour les protéines; mais en outre, leur présence confère à un grand nombre de substances d'importantes propriétés physiologiques. L'inhibition de la coagulation du sang, notamment, parait placée sous le contrôle de substances à fonction sulfonique [2, 4]. Les travaux de Hugget et Rowe [11] ont montré que certains colorants, le rouge Congo, le rouge et le bleu trypan notamment, possèdent des propriétés anticoagulantes. On peut concevoir des propriétés analogues pour le bleu à l'eau et le bleu de méthyle, qui possèdent également des groupes sulfoniques.

L'existence d'un système physiologique intracellulaire sensible aux substances anticoagulantes repose actuellement sur de nombreuses données. La présence, à l'intérieur des cellules, de substances actives sur l'état physicochimique des protéines, préalablement invoquée par Heilbrunn [8] et Runnström [20], a été démontrée dans des organismes et des tissus très variés [16, 8, 9, 23]. Selon la conception de Monné et Hårde [15], il existerait à l'intérieur des cellules deux systèmes physiologiques antagonistes, l'un à action coagulante, l'autre à action liquéfiante. Les variations d'équilibre entre ces deux systèmes seraient responsables des altérations structurales au cours du développement.

Des modifications structurales importantes interviennent également au cours de la détermination embryonnaire. Une indication en est donnée par les variations de la viscosité intracytoplasmique et de la rigidité corticale,

au cours des modifications expérimentales de la détermination [22, 12]. Dans une série de recherches, Ranzi et ses élèves [17] ont mis en évidence la variation de viscosité des protéines fibreuses en présence de divers agents animalisants et végétativisants.

Ces considérations nous permettent d'envisager une action directe des colorants étudiés sur l'ultrastructure intracytoplasmique. Ces remaniements structuraux seraient à l'origine des animalisations observées.

RÉSUMÉ

Les effets de différents colorants sur le développement de l'œuf de l'oursin *Paracentrotus lividus* sont décrits. Les colorants étudiés appartiennent d'une part, à la série azoïque : bleu et rouge trypan, rouge Congo, et d'autre part à la série du triphénylméthane : bleu à l'eau, bleu de méthyle. Les embryons cultivés en présence de ces colorants sont animalisés. L'effet animalisant de ces substances est discuté en fonction de leurs propriétés anticoagulantes, liées à la présence de groupes sulfoniques fixés sur leur molécule.

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MACRONUCLEAR AND NUCLEOLAR DEVELOPMENT IN *PARAMECIUM BURSARIA*[1]

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The nuclear events associated with conjugation and autogamy in *Paramecium* have been intensively investigated for over sixty years. Important recent contributions have been made by Chen [4, 5, 6], Diller [9, 10, 11] and for other ciliate genera by Seshachar [35, 36, 37]. Pertinent general reviews have been presented by Sonneborn [38], Grell [21], and Devidé and Geitler [8].

The present paper deals particularly with the disappearance of the old macronucleus and the appearance of the new, during, and immediately following, conjugation. Particular emphasis is placed on the origin and fate of macronuclear nucleoli throughout morphogenesis and vegetative fission.

MATERIALS AND METHODS

Clonal cultures of chlorella-containing *Paramecium bursaria* were grown on *Aerobacter cloacae* in dried lettuce broth [39] or in modified [30] Knop's solution. The stock designation of a particular specimen is given in the figure legends. Pineville (North Carolina) Variety 1 and Bat Cave (North Carolina) Variety 2 stocks were collected by the authors in 1953. All of the other stocks are Variety 1 and were generously provided by Professor T. T. Chen, University of Southern California.

Animals of complementary mating types were mixed during their early reactive periods [15] and conjugants were isolated from the reaction mixtures four to six hours later. The time of separation of conjugating animals into exconjugant pairs was recorded, and the split pairs were transferred into separate isolation depressions. The approximate duration of mating and time of separation were thus known for each pair. Room temperature was 25 $\pm$ 1°C.

Methods of observation were by phase contrast (Leitz) and electron microscopy (RCA type EMU 2A). In the former method squash preparations of unfixed animals were used. For the electron micrographs, animals were fixed in 1 per cent osmium tetroxide, pH 7.4, and sectioned in methacrylate by a glass blade with an International Minot Rotary microtome at thicknesses of 0.05–0.10 μ. After being mounted on formvar covered grids, the sections were soaked briefly in toluene to remove the plastic.

[1] Work performed under the auspices of the U.S. Atomic Energy Commission.

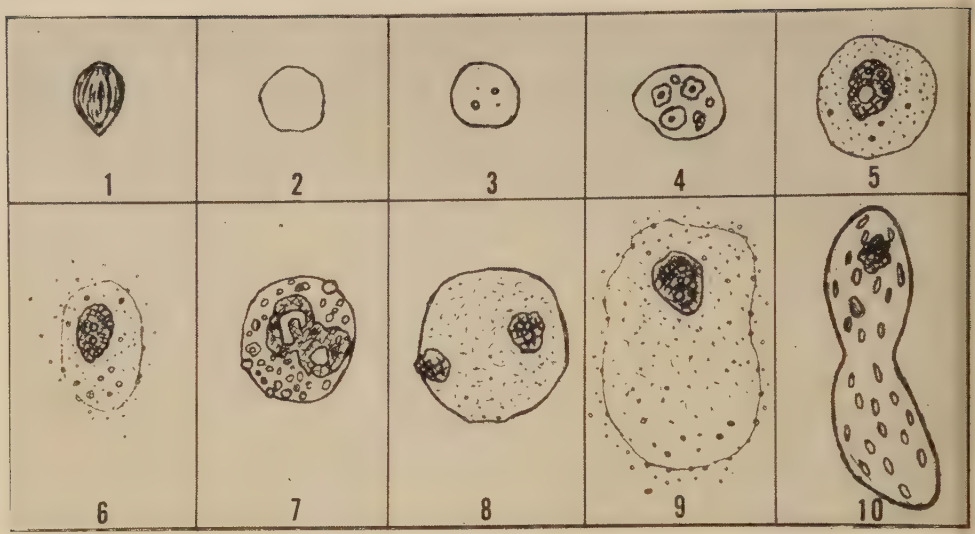

Diagram I. A generalized description of the stages of macronuclear development: 1—microform; 2—swollen form; 3—first nucleolar generation; 4—nucleolar enlargement; 5—second nucleolar generation; 6—nucleolar extrusion; 7-nucleolar enlargement; 8—third nucleolar generation; 9—nucleolar extrusion; 10-nucleolar enlargement; (11—vegetative interphase).

OBSERVATIONS

Vegetative macronucleus. –The gross appearance of the vegetative macronucleus and that of the micronucleus are shown in Fig. 2. The macronucleus in the living condition consists of a fluid matrix surrounded by a membrane. In this fluid there occur countless very small granules which give the macronucleus its fine-grained appearance in phase photographs. In addition, optical sections through it reveal over seventy randomly distributed "large bodies" [16] identified on the basis of appearance and cytochemical reactions as nucleoli [17], and probably homologous with the "nucleolar" bodies of Bretschneider [2] and Kimball [26]. These bodies are Feulgen negative, stain pale blue-green with Janus Green B, pink with 2,3,5-triphenyl tetrazolium chloride, dissolve in dilute acetic acid and in sodium hydroxide to give the "Swiss-cheese effect" [17]. The "Swiss-cheese effect" is seen in Fig. 3, which pictures the same macronucleus as that shown in Fig. 2, after staining with acetocarmine. A one-to-one correspondence between the "holes" or "vacuoles" (described as such in the fixed animals of the older literature) and the nucleolar loci can be easily demonstrated in a series of optical sections. The micronucleus of most ciliates is apparently devoid of such nucleoli; instances of micronuclear nucleoli have been described in the *Opalinidae*

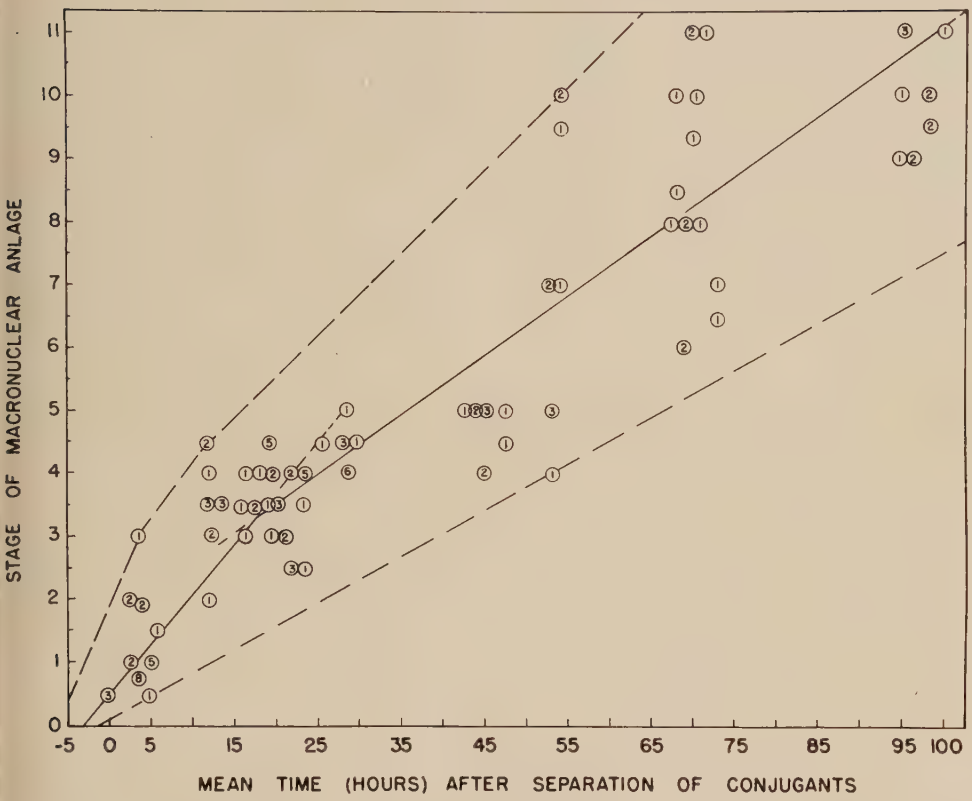

Diagram II. The relationship between stage of macronuclear anlage and time at which observation was made, as measured from conjugant-separation time. Average conjugation time was 24.1 hours for Variety 1 (B × D) animals at 25°C. The encircled figures refer to the numbers of animals observed at each point.

which, in addition, differ from most other ciliates in possessing no macronucleus [7].

Development of the macronucleus.—Following conjugation and the early post-zygotic divisions of the syncaryon, there can be observed a definite sequence of events leading to the vegetative nucleus from the macronuclear anlage. Eleven morphologically distinct stages of macronuclear development are recognized, the first being the microform nucleus indistinguishable from a micronuclear anlage, and the last being the fully developed vegetative macronucleus. Diagram I is a representation of the stages which are described verbally below. Stages have been abstracted from observations of over 550 exconjugants of Variety 1, and of Variety 2 crosses.

Beginning with animals just after pairing, the sequence is as follows: the micronuclei undergo three pre-zygotic (two meiotic, one equational) divisions, exchange of pronuclei is accomplished, and the zygote nucleus (syncaryon) is formed in each conjugant. Fig. 4 shows such a syncaryon undergoing the first post-zygotic division. Chen [4, 6] and Wichterman [40] have described a degeneration of one product of this first division, and conclude that the four anlagen nuclei arise from the third post-zygotic division. In most instances (~ 90 per cent), we believe this does not occur in these particular stocks, and we tentatively conclude, as did Hamburger [22], that the syncaryon divides twice, giving rise to four small and morphologically similar nuclei. Later two of these become swollen, two remaining small (Figs. 5, 6 and 7). The determination is not clearly related to position in the cell. Within each swollen macronuclear anlage small dots (ca. 0.2μ diameter) appear, which enlarge until they identify themselves structurally and cytochemically as the familiar nucleoli (Fig. 8). Nucleoli of this first nucleolar generation enlarge (Fig. 9), and fuse to form a net-like agglomerate [12, 18] at the same time those of the second nucleolar generation make their appearance (Fig. 10).

In these preparations, about 30 seconds after compression, a space separating the macronucleus and the cytoplasm appears. The macronucleus is bounded by its membrane of the sort represented in Fig. 36. The cytoplasm appears to be bounded by an interface which grows as the invasive fluid of the

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Abbreviations used in the figures:

mc—macronucleus (and old macronucleus); mi—micronucleus; mca—macronuclear anlage (and new macronucleus); mci—micronuclear anlage; mcm—macronuclear membrane; n—nucleolus; no—old nucleolus; nt—net-like nucleolar agglomerate; m—mitochondria; chl—chlorella; cy—cytopharynx.

Fig. 1. *Paramecium bursaria*, demonstrating gross structural relationships in the living animal. Stock Pineville (Pi) 6–5. The 20-micron scale indicates magnification.

Fig. 2. Micronucleus, macronucleus and nucleoli of one optical section are shown; the overlying and obscuring pellicle has been removed by compression-rupture of the animal. Depth of field is about 40 micra. Pi 6 5. The 10-micron scale indicates magnification, which is the same for Figs. 2–35.

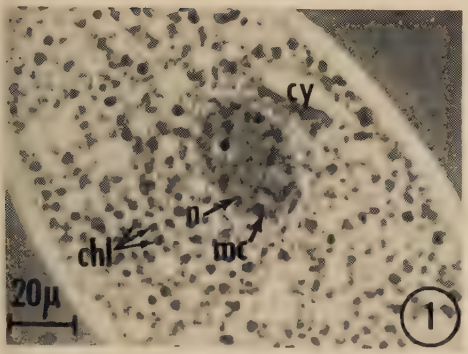
Fig. 3. Same specimen as preceding, stained with acetocarmine to show the nucleolar loci of the "Swiss-cheese effect".

Fig. 4. First post-zygotic division in one member of a pair still fused in conjugation. Bat Cave (BC) 1–4 × BC 1–10.

Figs. 5, 6. Second post-zygotic division, early stage 2 macronuclear anlagen in Fig. 5 A photomontage from a recent exconjugant. BC 1–4 × BC 1–10.

Fig. 7. Stage 2 macronuclear anlagen; a nearly normal-appearing old macronucleus is also visible. BC 1–4 × BC 1–10.

Fig. 8. Stage 3 macronuclear anlage, showing the early distribution of young nucleoli. So 52 × Wu 5. Figures 8–35 represent crosses of these same stocks.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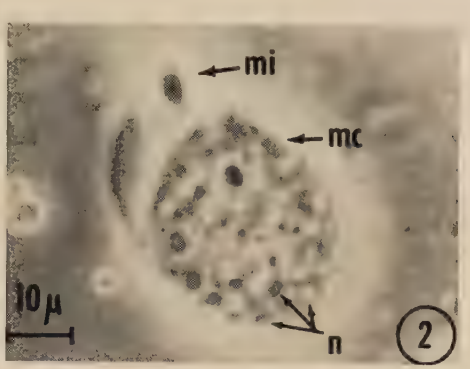
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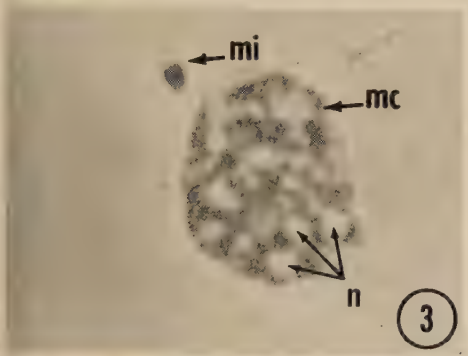
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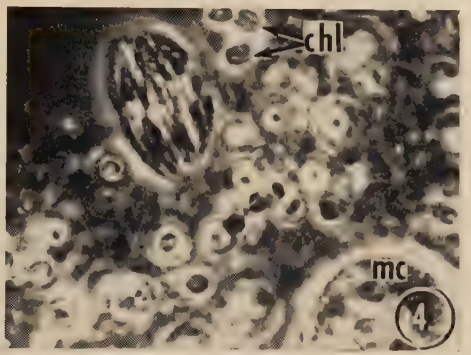
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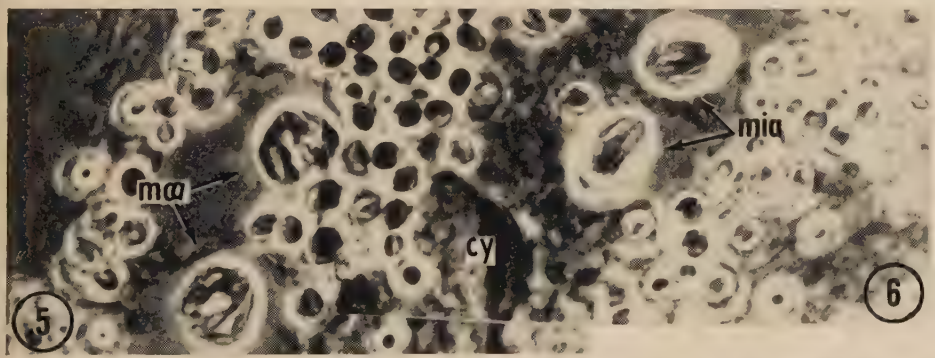
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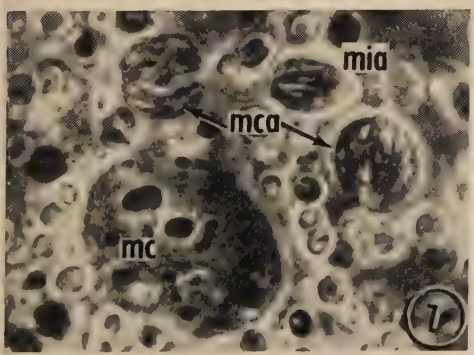
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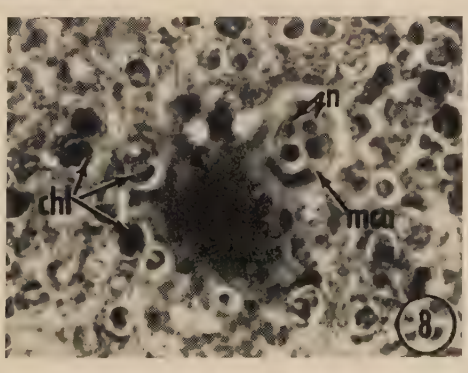
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perinuclear space increases in volume. Electron-micrographic evidence in our preparations for "doubleness" of nuclear membranes [23, 34] is at best inconclusive. Nucleolar extrusion through the nuclear membrane into the perinuclear space is evident at this stage and continues until the nucleoli are about two micra in diameter (Fig. 11). The nucleoli of the second nucleolar generation that remain within the nucleus enlarge and fuse to form a net-like structure (Fig. 12), and those of the third nucleolar generation arise as barely resolvable dots (Fig. 13). Nucleolar extrusion (Figs. 14, 15) again occurs, followed now by enlargement of nucleoli (Fig. 16) and cytokinesis, sometimes accompanied by macronuclear anlage division and somtimes not.

Since two macronuclear anlagen were present at stage 1, each of the two daughter cells will have two macronuclei if anlagen divisions occur before cytokinesis (Fig. 17), and only one macronucleus each if anlagen divisions do not occur before it (Fig. 18). Both phenomena appear to occur with nearly equal frequency, although the precise statistics are presently unknown to us. An example of the second case (cytokinesis before further macronuclear division) is shown in Figs. 19–20, representing two caryonidal daughter cells, each with a single micronucleus and macronucleus.

The time sequence of the events following the conjugation of mating types B and D of Variety 1 (stocks So 52, Wu 5) at 25°C is described in Diagram II. In this figure the stages indicated are as represented in Diagram I. Stages 0 and 0.5, not shown in Diagram I, are the syncaryon and the products of the first post-zygotic division respectively. The average time between each stage is about 6.2 hours from stages 0 to 3, and about 10.5 hours from stages 3 to 11, although the stage to stage progression of an individual is probably continuous. The large spread at any particular stage should be noted, as indicated by the dotted lines in the figure.

Fate of the old macronucleus.—The fate of the old macronucleus as a reticulated remnant, which shrinks until it disappears (Figs. 19 and 21) in the

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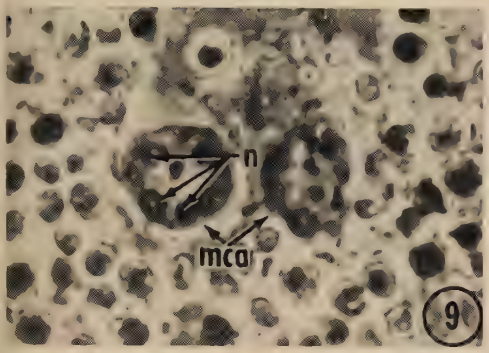
Fig. 9. Stage 4, showing first evident increase in size of macronuclear anlagen.
Fig. 10. Stage 5 anlage early in the second nucleolar generation showing net-like figure resulting from coalescence of nucleoli of first nucleolar generation.
Fig. 11. Stage 6 7 anlage showing late extrusion of large young nucleoli of second nucleolar generation.
Fig. 12. Stage 7 anlage, in which fusion of nucleoli of second nucleolar generation has begun.
Fig. 13. Stage 8, early in the third nucleolar generation; the net-like agglomerate is the remnant of the second nucleolar generation.
Fig. 14, 15. Stage 9, active third generation nucleolar extrusion; the anlage of Fig. 14 is apparently membraneless.
Fig. 16. Stage 9–10; nucleolar enlargement and early suggestion of division; old macronucleus becoming degenerative.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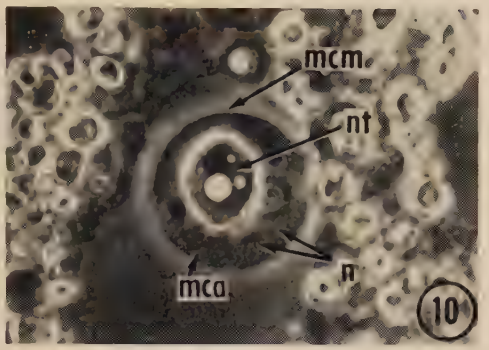
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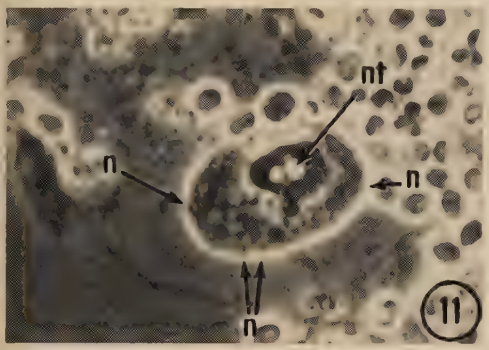
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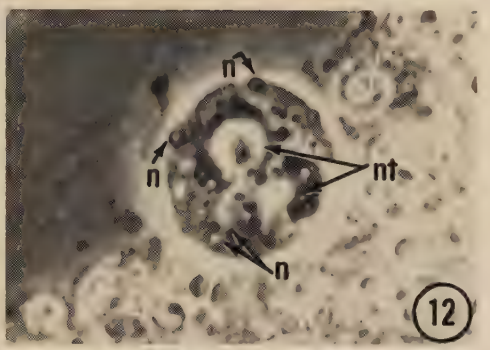
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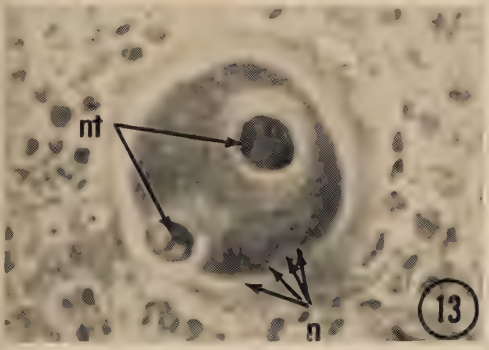
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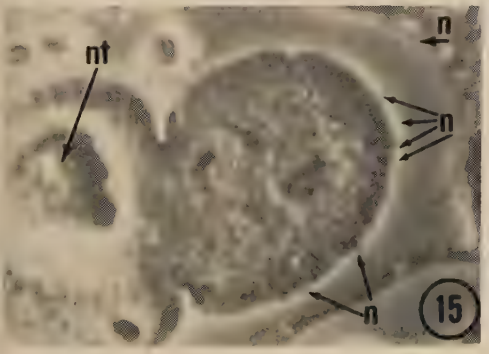
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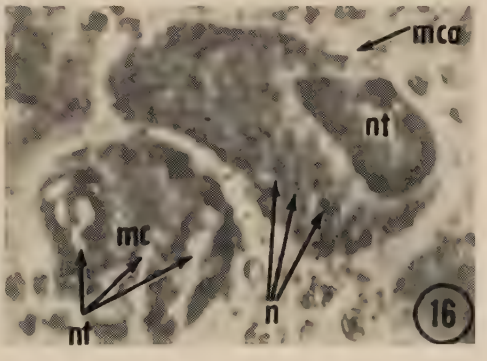
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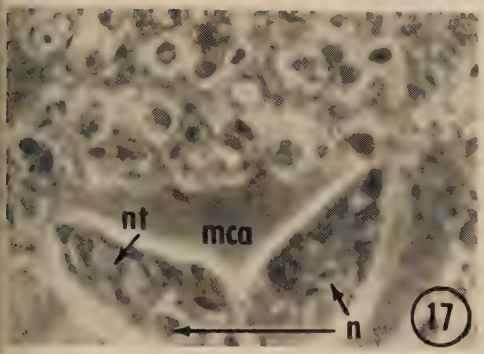
anterior daughter cell (protisthe), is evident at an exconjugant's first division. The reticulum is devoid of nucleoli, and appears threadlike and empty following acetocarmine staining (Fig. 22). Earlier in the process, the changes in the old macronucleus leading up to this state are as follows. Prior to cytokinesis nucleolar extrusion is evident at the second post-zygotic division (stage 1 or earlier) of both exconjugant (Fig. 23) and exautogamous (Fig. 24) [5] animals. Net-like formations similar to those in the newly developing macronucleus arise during stages 2–3 (Fig. 25), and nucleolar extrusion occurs during stages 3–4 (Fig. 26), stages 5–6 (Fig. 27) and stages 8–9 (Fig. 28). After this no further nucleolar activity is observed, and the old macronucleus with its nucleolar net gradually shrinks as noted previously. At this time the kinetosomal field [27] of the new cytopharynx is seen, and shortly thereafter the exconjugant animal divides for the first time (stages 10–11).

Nucleolar behavior during fission.—The nucleolar activity associated with the division of a typical vegetative nucleus (Fig. 2) is demonstrated by Figs. 29–35. Net-like coalescence of nucleoli occurs (Fig. 29) followed by nucleolar extrusion (Figs. 32 and 33) and enlargement (Figs. 30 and 35) and macronuclear division. During these events the micronucleus is in early prophase (Fig. 29), metaphase-anaphase (Fig. 32), telophase (Fig. 31), and late telophase (Fig. 34) respectively. It may be noted that at the time of nucleolar extrusion a thin-membraned or apparently membraneless condition sometimes exists (Figs. 33 and 14). The pre-fission animal appears characteristically swollen during micronuclear prophase-anaphase, and cytokinesis is not externally evident until micronuclear anaphase-telophase.

Ultrastructure of nuclear components.—Electron micrographs of thin sections of vegetative animals show that the nucleoli are complex structures 0.6 μ or more in diameter, with fine-grained centers and densely osmiophilic perimeters (Fig. 36). There is no visible attachment to the small granules in the nuclear matrix, and superficially little resemblance to any cytoplasmic structure. Since the observed extrusions of *young* nucleoli imply the simul-

Figs. 17, 18. Stage 10, nucleolar enlargement, and division in Fig. 17; no division, possibly re-fusion in Fig. 18.
Figs. 19, 20. Stage 11 protisthe and opisthe caryonides; the former daughter cell contains the old macronuclear remnant.
Figs. 21, 22. Barely discernible remnant of old macronucleus before and after staining with acetocarmine. Note the net-like architecture and absence of nucleoli. See Figs. 2 and 3.
Fig. 23. Extrusion of young nucleoli from old macronucleus of exconjugant during anaphase of second post-zygotic division.
Fig. 24. Extrusion of young nucleoli from old macronucleus of exautogamous ("third") animal during anaphase of second post-zygotic division.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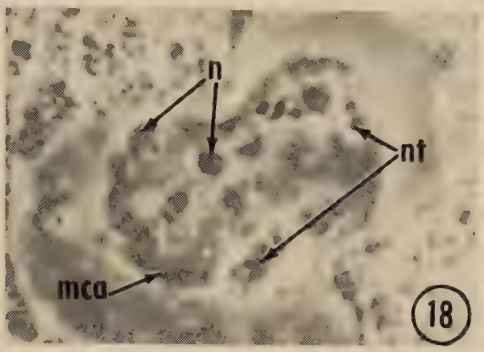
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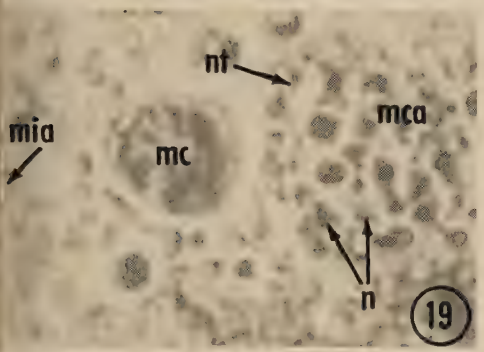
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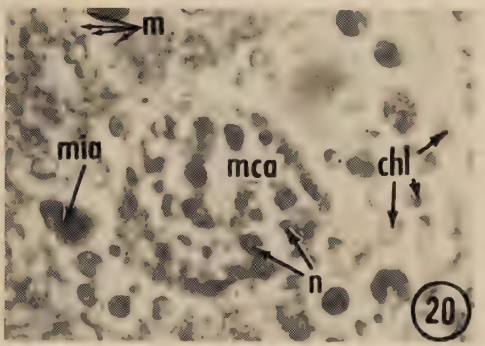
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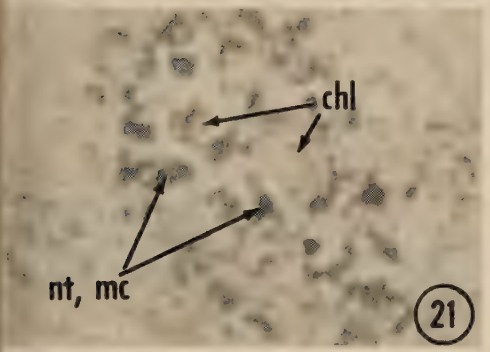
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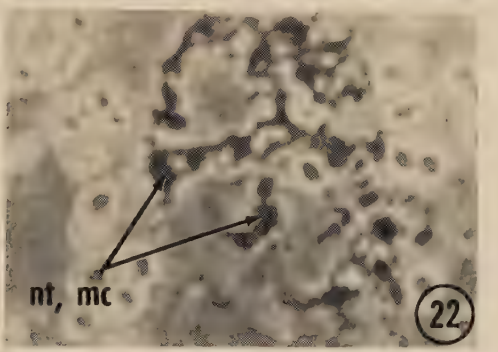
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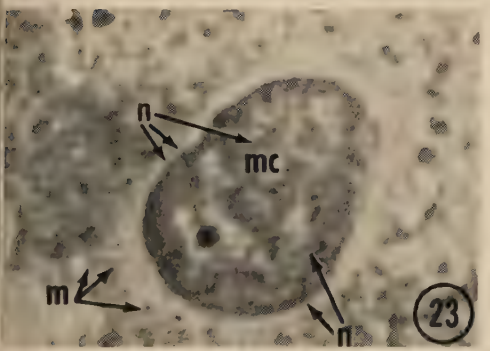
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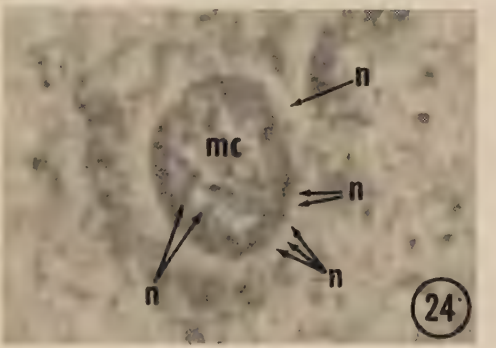
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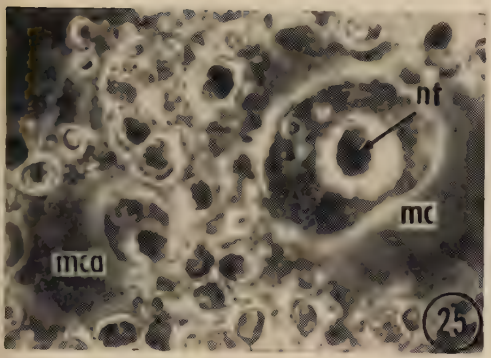
taneous existence in the nucleus and cytoplasm of isomorphic units, the structure of *young* nucleoli was sought on electron micrographs. Exconjugants in the early 2nd post-zygotic division stage (Fig. 23) were sectioned, with the results shown in Figs. 37 and 38. The young nucleoli (about 0.5 μ or less in diameter) are clearly evident in both sections, and appear identical with some of the cytoplasmic particulates present. The latter are similar to and indeed usually indistinguishable from the previously identified compact mitochondria [32, 33] of *Paramecium bursaria*. Since it has been observed that some nucleoli as small as 0.2 μ diameter are extruded, it is possible to consider enlargement of these within the cytoplasm by growth or coalescence, although we as yet have no such direct evidence.

A section of a macronuclear anlage (stage 3) with a young nucleolus within the fine-grained matrix is seen in Fig. 39; the old macronucleus is seen to have both young nucleoli (0.2–0.3 μ diameter) and the net-like figure present simultaneously, as demonstrated above in the unfixed preparations (Figs. 25, 26).

DISCUSSION

It has been demonstrated that during particular steps of macronuclear generation and of vegetative fission, the nucleoli of *Paramecium bursaria* arise within the macronucleus from very small bodies. Some of these are extruded into the cytoplasm from early, mature, and degenerative stages of the macronucleus; the nucleoli remaining within the macronucleus enlarge (apparently by means of both growth and coalescence, Fig. 36) and fuse finally into a net-like form which finally disappears, shortly after the next nucleolar generation arises. Numerous speculations have been made regarding the apparently passive contribution to the phenotype by the old macronucleus, whether it be by its gradual disappearance as in *P. bursaria*, or by skein or fragment formation as in other species (*P.*

Fig. 25. Old macronucleus at anlage stage 3–4, showing a net-like figure, and mature nucleoli.
Fig. 26. Thin-membraned active old macronucleus, showing young nucleoli and old net-like structure during anlage stage 4–5.
Fig. 27. Old macronucleus during anlage stage 6 7 showing formation of net-like structure and late nucleolar activity.
Fig. 28. Final extrusion of young nucleoli from old macronucleus, during anlage stage 9.
Figs. 29, 32, 33, 30, 31, 34, 35. Chronological sequence of vegetative fission. Pi 6–5.
Fig. 29. Coalescence of nucleoli into net-like structure; enlargement (early prophase) of micronucleus.
Figs. 30, 31. Discrete young nucleoli, intact macronuclear membrane, and micronucleus in telophase.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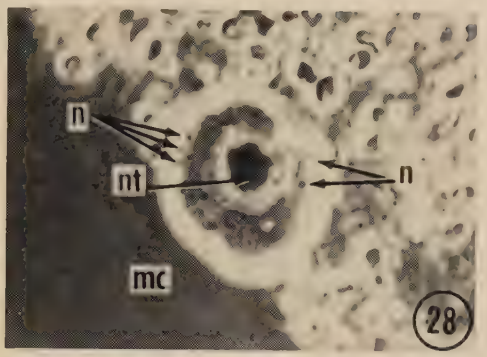
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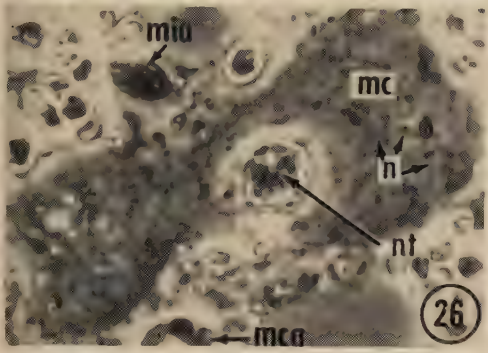
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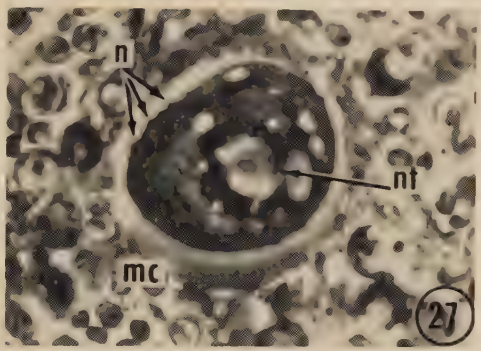
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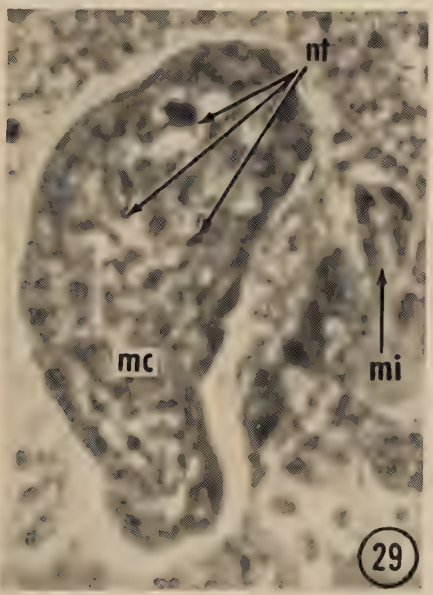
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aurelia, P. trichium, P. polycaryum) [9, 10, 11]. It is now evident that prominently associated with macronuclear disappearance is an *active* extrusion of material into the cytoplasm, a process not unlike that occurring in the anlagen during development, or in the vegetative macronucleus during fission.

The synchrony of nucleolar generation and extrusion in the two anlagen and the old macronucleus suggests a control arising outside the nuclei. In addition, the constant relations one sees between stages of micronuclear division and macronuclear (nucleolar) activity suggest that the micronuclei are in the same control system. Nucleolar generation is usually correlated during fission with micronuclear metaphase-anaphase, and during both fission and post-zygosis with extremely thin and possibly absent macronuclear membranes (stages 6, 9; Figs. 11, 14, 23, 24, 26, 32, 33, 37, 38, 39) in contrast to the drum-tight thick appearance of the membranes at interdivision stages.

Historically, the entire subject of extrusions from the nucleus is too lengthy to consider here, and several older reviews may be consulted [19, 28]. Observations in the ciliates include that of Kidder and Claff [25] who observed, in fixed preparations, nuclear extrusions in *Colpoda*, concluding from this and the extensive observations of other authors that elimination of material from the ciliate macronucleus may occur universally. Even more recently Diller [11] reported that in exautogamous cells of *Paramecium polycaryum* one sees granules bulging from the central mass of the anlage substance "as if they were being extruded". The Hertwig chromidial hypothesis (and Goldschmidt's version of this [20] with Dobell's exceptions [13] to its binuclearity aspects) and its subsequent unpopularity should also be recalled. Dodson [14] has recently reappraised the binuclearity theory and proposes that along the chromosome (idiochromatin) there exist sites of RNA synthesis (trophochromatin); and that the nucleolar proteins which are produced at these sites are secreted into the cytoplasm and represent the developmental enzymes of the cell.

The clearest demonstration of ciliate nucleoli is that of Chen [7] in the amacronucleate and astomatous opalinid, *Zelleriella*, in which there was

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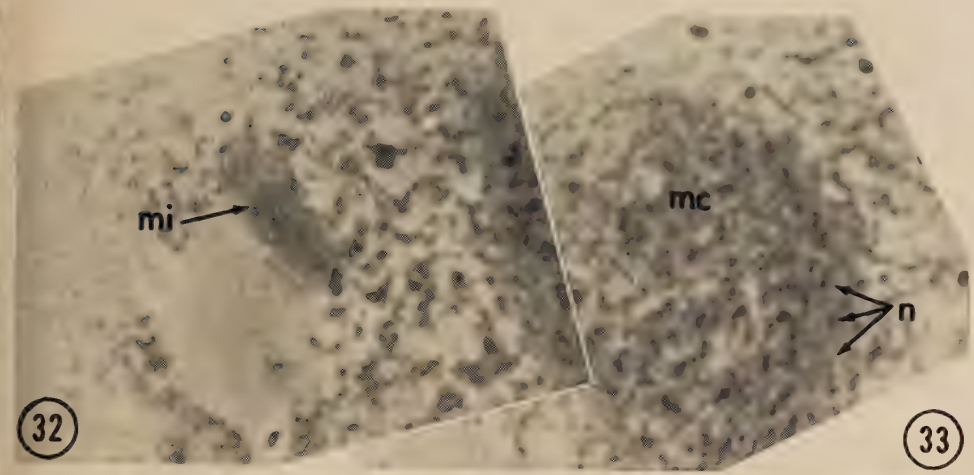
Figs. 32, 33. Generation and extrusion of nucleoli, nearly membraneless macronucleus; micronucleus in meta-anaphase.
Figs. 34, 35. *Sigma*-shaped figure of early interphase micronuclei, separating into the fission daughter cells; macronucleus in evident "amitosis" with intact membranes and enlarged young nucleoli.
Fig. 36. An electron micrograph which demonstrates the appearance of the micronucleus, macronuclear matrix, mature nucleoli, and mitochondria during interphase.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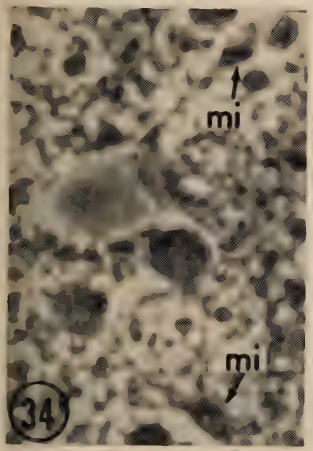
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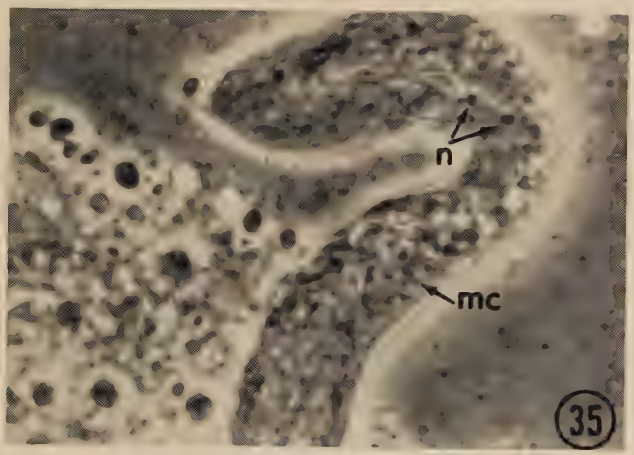
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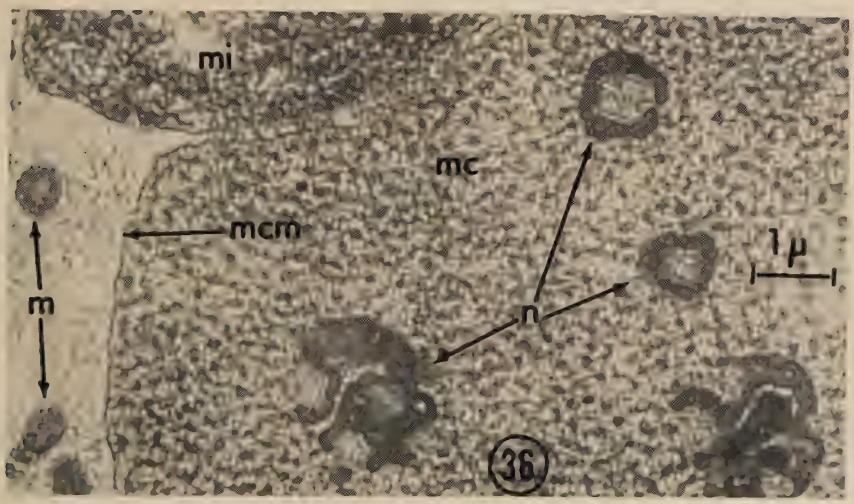
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shown not only a specific association between the chromosomes and nucleoli, but also variable patterns and frequencies of nucleolar fusion and detachment, the latter forms suggestive of both the old and the net-like nucleoli of *P. bursaria*.

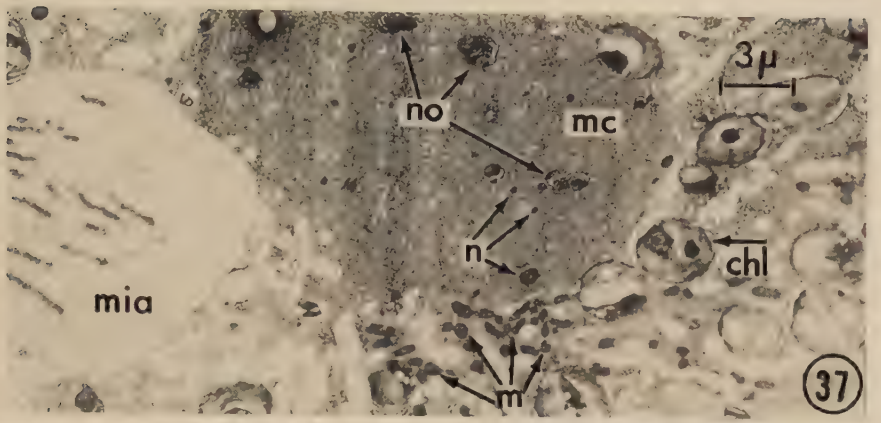
The macronuclear nucleolus itself has probably eluded critical identity until now [26] largely on account of its lability in the presence of the fixatives which have been employed. In addition, perhaps its true nature has been overlooked because of possible confusion with nuclear parasites. For instance, in 1904 Calkins [3] described bodies in the macronucleus of *P. caudatum* as a sporozoon *Caryoryctes cytoryctoides*, although the figures describing them can serve as illustrations of net-like nucleoli. However, it is interesting to note in this connection that Calkins' interpretation was based partly on their resemblance to the "Guarnieri" bodies (*Cytoryctes variolae*—thought then by some to be the causative agent of smallpox), that smallpox was subsequently demonstrated to be a virus disease, that the invasion of the nucleus by viruses has been described [29], and that, previous to this, Paschen's [31] considerations of the origin of "Guarnieri" bodies includes that of extruded nucleoli. While the interrelations among all these observations and suggestions are far from clear today, such considerations of pathologic nuclei may contribute to our notions of nuclear structure and function.

The observations of isomorphism (Figs. 37, 38) between the *young* nucleoli and the mitochondria [33] in animals *sectioned at the very time of nucleolar generation and extrusion* (Figs. 23, 24) is suggestive evidence for the intranuclear origin of these mitochondria. There is in addition a remarkable ultrastructural resemblance between the *old* nucleoli of Fig. 37 and the nebenkern of the grasshopper [1], which has been shown to arise by the fusion of mitochondria. The further evolution and differentiation of these bodies (nucleoli and mitochondria) are being investigated.

SUMMARY

1. The development of the macronucleus and nucleolus in *Paramecium bursaria*, studied by means of phase contrast and electron microscopy, has been described.

Figs. 37, 38. Electron micrographs showing isomorphism of cytoplasmic particulates and young nucleoli in old macronucleus of exconjugant during the second postzygotic division (Figs. 23 and 24 above). Note the nebenkern-like old nucleoli in Fig. 37. So 52 × Wu 5.
Fig. 39. Electron micrograph showing stage 2 -3 macronuclear anlage with fine matrix and young nucleoli, and coarse matrixed old macronucleus, containing young nucleoli and net-like figure. So 52 × Wu 5.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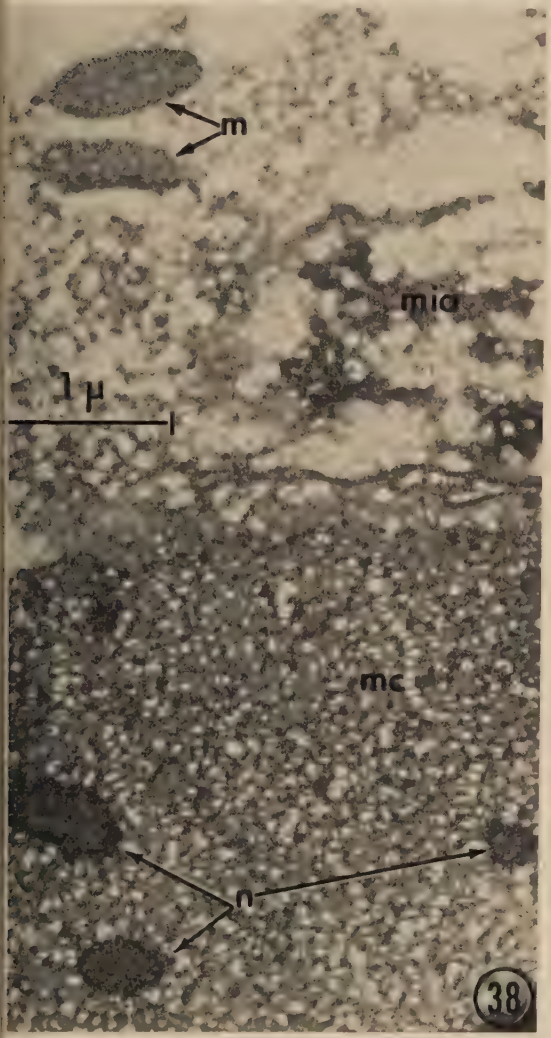
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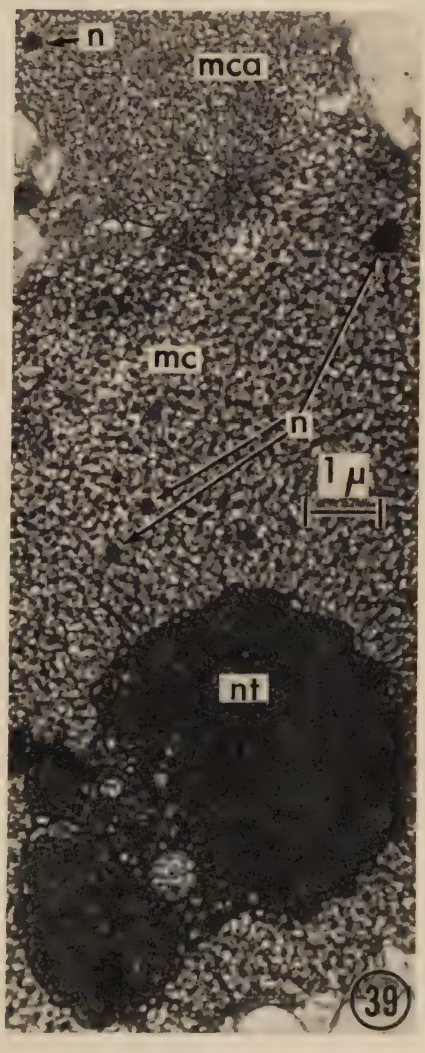
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and mounted in Euparal Vert (Flatters and Garnett). Haemalum stains the nuclear DNA dark blue and the nucleolar and cytoplasmic RNA a purplish grey. Assessment of cytoplasmic basophilia is very precise because haemalum does not overstain nor does it wash out. In a few experiments cultures were fixed in Zenker-formol, sectioned in low viscosity nitrocellulose at 4μ, and the sections stained with Haemalum, Giemsa or Windle's buffered thionine at pH 5.3

Quantitative Assessment of Results

The various cell types found in the film preparations are shown in Fig. 1. In normal nodes there are two main categories of cells, the reticulum cells (*R*) and the lymphocytes, the latter being subdivided, after Maximow, into large (*L*), medium (*M*) and small (*S*1–5) lymphocytes. It is generally accepted that the normal process of lymphopoiesis is reticulum cell →large lymphocyte →medium lymphocyte →small lymphocyte, with cell divisions occurring in all except the small lymphocytes. It may save confusion to point out that the cells in peripheral blood called 'large lymphocytes' by haematologists correspond to the medium lymphocytes of the Maximow terminology used here. Large lymphocytes (of Maximow) do not normally occur in peripheral blood and they correspond to the lymphoblasts and haemocytoblasts of other terminologies.

According to many authors, the reticulum cells must be further subdivided into 'primitive reticulum cells' which are lymphopoietic and 'phagocytic reticulum cells' which are specialised end-cells belonging to the reticulo-endothelial system. We believe however, on evidence to be mentioned later, that all the reticulum cells are both phagocytic and potentially lymphopoietic.

The lymph nodes used in this work show very little lymphopoiesis even in the intact animal, germinal centres are usually absent and the nodes consist essentially of diffuse lymphoid tissue. In film preparations of normal nodes the average proportions of small, medium and large lymphocytes are 99.2, 0.7, 0.1 per cent respectively and about 1 per cent of the medium plus large lymphocytes are in mitosis.

The film preparation from each culture was assessed from three different points of view, as follows:–

(i) *Pyknotic count.*—When lymphocytes die their nuclei become pyknotic and this is easily recognised in the film preparations. The normal and pyknotic lymphocytes were counted in each of a series of microscope fields to a total of over 500 cells and the percentage of the lymphocytes which were pyknotic (the Pyknotic Count) determined. The counts of the 15–20 cultures in each culture chamber were averaged to give a mean figure with a standard

Fig. 1. The types of cells found in lymph node cultures. Drawings made from film preparations wet-fixed in Susa and stained with Haemalum. *R*, reticulum cells; *RL*, cells intermediate between reticulum cell and large lymphocyte; *L*, large lymphocytes; *M*, medium lymphocytes; *Mon*, monocytes; *S*, small lymphocytes (grades 1–5).

2. Eleven morphologically distinct stages in macronuclear development have been recognized, beginning with the micronucleus-like microform and terminating in the vegetative interphase form.

3. Within the macronuclear anlagen at least three waves of nucleoli (nucleolar generations) have been observed, during anlage stages 3, 5, and 8 respectively.

4. Nucleolar extrusion from the macronuclear anlagen occurs during stages 6 and 9; the nucleoli extruded are those of the second and third nucleolar generations respectively.

5. Nucleolar generation and extrusion from the old macronucleus is synchronous with that observed in the anlagen.

6. Unextruded nucleoli enlarge by growth and by aggregation within the macronucleus. Later coalescence of nucleoli is associated with development of a net-like structure which disappears from the nucleus with an ensuing nucleolar generation.

7. A similar nucleolar cycle of generation, enlargement, and net-like coalescence is observed during vegetative fission, beginning prior to cytokinesis and during micronuclear prophase-metaphase.

8. The young nucleoli at the time of extrusion are similar in appearance cytoplasmic particulates previously described as mitochondria.

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error. The pyknotic count is a convenient measure of the lymphocyte survival under various conditions. It may be mentioned that the reticulum cells survived perfectly well in all the media tested, it was extremely rare to find a dead one.

(ii) *Cell quality* (lymphocytes).—The final appearance of the small lymphocytes depended very much on the medium in which they had been cultured. In good media the cells were relatively larger and more darkly staining than in poor media. In general, media which gave a low pyknotic count also gave good quality cells, but this parallelism did not always hold and it seemed desirable to have some separate assessment of 'cell quality' as an additional index of lymphocyte nutrition.

The good-quality small lymphocyte has a relatively large nucleus with plenty of densely staining chromatin and a prominent nucleolus, while the cytoplasm is relatively abundant and basophilic (Fig. 1, *S*1). The poor-quality small lymphocyte has a much smaller nucleus with sparser and less deeply staining chromatin, no nucleolus and no visible cytoplasm (Fig. 1, *S*5). In any one film the cells varied somewhat but it was possible to classify them on the average into one of five arbitrary grades, ranging from Grade 1, the best quality, to Grade 5, the poorest quality (Fig. 1, *S*1–5). Each film was graded in this way and the results were averaged to give a cell quality (grade) figure for the whole experiment.

This grading refers primarily to the small lymphocytes, but the few medium and large lymphocytes present showed parallel though less extreme variations, the most conspicuous of which were in the amount and basophilia of the cytoplasm and the size of the nucleolus. It is interesting to note that these are the cytological features which, according to Caspersson, reflect the rate of protein synthesis in the cell.

As will be shown later, with some of the best synthetic media the lymphocytes were finally bigger and better nourished than those of the normal lymph nodes. On the scale adopted, the lymphocytes of normal nodes only made an average grade of 2.5.

(iii) *Cell differentiation.*—In cultures which had been maintained for 4 days in good synthetic media there was evidence, firstly, of reticulum cells turning into large lymphocytes, and, secondly, of small lymphocytes turning into monocytes. Morphologically these two differentiation processes are quite unrelated, but in the cultures they nearly always went hand in hand, that is, media which promoted the one also promoted the other. These changes were sometimes found in minor degree in 2-day cultures, but they were not found after culture in serum, even for 6 days.

Reticulum cell → large lymphocyte.—The evidence for this was the finding in the films of many cells (Fig. 1, *RL*) which were intermediate in morphology between reticulum cells (*R*) and large lymphocytes (*L*). Such cells were not found in normal nodes. The morphological change involves a reduction in cytoplasm, increase of cytoplasmic basophilia, increase in size of the nucleolus and condensation of the nuclear chromatin. All stages of transition were present, that illustrated (*RL*) is a typical half-way stage. It must be admitted however that the percentage of genuine large lymphocytes in the cultures did not rise significantly above the starting figure and it seemed that many of these transforming cells were arrested at a stage in which the nucleus was typical of a large lymphocyte but the cytoplasm was still a little too copious and slightly vacuolated. In sections it was seen that in quite large focal areas all of the reticulum cells were in some stage of this transformation, and, taking the culture as a whole, often more than half of the reticulum cells were transforming. This observation, together with the fact that we have sometimes seen phagocytosed pyknotic debris (from dead lymphocytes) in the cytoplasm of cells which had almost reached the stage of large lymphocytes, is the basis for our belief that it is the ordinary phagocytic reticulum cells which turn into large lymphocytes. It is interesting to note that in 1923 Maximow [7] reached a similar conclusion though he later returned to a belief in primitive reticulum cells. The observed changes cannot be interpreted in the reverse way as a transformation of large lymphocytes into reticulum cells, for there are usually far more transforming cells present than there ever were large or medium lymphocytes in the original nodes. Mitoses in these cultures are so few as to be negligible in this connexion.

In normal lymph nodes new lymphocytes are mainly produced by multiplication of medium and large lymphocytes (homoplastic lymphopoiesis), there is little or no new formation of large lymphocytes from reticulum cells. The phenomenon observed in these cultures may be regarded as the first stage in heteroplastic lymphopoiesis, a process which usually only occurs in the regeneration of lymph nodes after destruction of all the lymphocytes by radiation, nitrogen mustard, cortisone, etc.

Small lymphocytes → monocytes.—The evidence for this was the finding in the films of cells which were identified as monocytes and of all transitions between small lymphocytes and monocytes. These cells (Fig. 1, *Mon*) were about the size of a medium lymphocyte but the cytoplasm was much less basophilic and the nucleus was distinctly different, having a finer chromatin pattern, a smaller nucleolus and a thinner nuclear membrane which was often irregularly indented. These cells looked exactly the same as the blood

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monocytes which were studied in similarly made films from the buffy coat of blood from the same rats. When supravitally stained with neutral red they showed a clump or rosette of fair sized granules at the nuclear 'hof', whereas normal lymphocytes showed only a few tiny scattered granules. Bloom [2] has stated that rat lymphocytes sometimes show a neutral red rosette but we have not found this to be the case in our normal lymph nodes. Supravital staining with pinacyanol showed that these cells contained about twice as many mitochondria as normal small lymphocytes. When trypan blue (0.5 per cent) was added to the culture medium two days before the end of the experiment, many of these cells segregated a certain amount of the dye whereas the ordinary lymphocytes did not.

In many cultures 10 per cent or more of the free cells were monocytes and, in the virtual absence of mitosis, it seemed clear that they could only have arisen from small lymphocytes as no other cells were present in sufficient number. In sections of cultures it was seen that the small lymphocytes which collected in the lymph sinuses never turned into monocytes, it was only those in the reticular tissue network that did so.

The hypertrophy of small lymphocytes into monocytes and macrophages has of course been recorded before in cultures of buffy coat, lymph, lymphoid tissue and also in inflammation, and the whole subject has been reviewed by Rebuck [11].

The extent to which these cell differentiations had occurred was assessed for each experiment (chamber) as a whole and recorded by a system of scoring, ranging from — to + + + +.

RESULTS

The lymph nodes were cultured for either 2 days or 4 days and in the 4-day experiments the medium was removed and replaced with fresh after two days. The general plan was to start with Tyrode solution and add to it, step by step, simple nutrients such as amino acids, vitamins, coenzymes, purines and pyrimidines, etc. In this way it was hoped to build up a synthetic medium which might be as good as the rat serum used in previous work. In the event the medium finally developed proved to be better than serum. Results obtained with serum (plus extra glucose) in 2-day and 4-day experiments are shown in Tables II and III and it is seen that although the lymphocytes survived very well, the pyknotic count remaining below 2 per cent, the cell quality was distinctly poor and there was no cell differentiation. Table II also gives figures for normal lymph nodes.

Modified Tyrode Solution (Medium T)

Ordinary Tyrode solution gives poor results, chiefly because it contains insufficient glucose. A modified Tyrode solution however gave results which were surprisingly good considering that glucose was the only nutrient pro-

TABLE I
Composition of Medium *TACPI*.

	mM/l	mg/100 ml
NaCl	104	610
KCl	6	45
$CaCl_2$	2	22
$MgSO_4 \cdot 7H_2O$	1	25
$NaH_2PO_4 \cdot 2H_2O$	1	15
$NaHCO_3$	33.5*	282
Glucose	22	400
Chloramphenicol	0.1	3.0
Phenol Red	0.03	1.0
L-Glutamic Acid	1.0	14.7
L-Leucine	1.0	13.1
Glycine	0.5	3.8
L-Valine	0.5	5.9
DL-Serine	0.5	5.3
DL-Threonine	0.5	6.0
L-Lysine. HCl	0.5	9.1
L-Arginine. HCl	0.5	10.5
L-Aspartic Acid	0.5	6.7
Lα-Alanine	0.3	2.7
DL-Phenyl Alanine	0.3	5.0
L-Tyrosine	0.3	5.4
L-Isoleucine	0.3	3.9
L-Proline	0.3	3.5
L-Cysteine. HCl	0.3	4.7
L-Histidine. HCl	0.1	1.9
L-Hydroxyproline	0.1	1.3
L-Tryptophane	0.1	2.0
DL-Methionine	0.1	1.5
Cocarboxylase	0.05	2.4
p-Aminobenzoic acid	0.25	3.5
Insulin	0.001	5.0

* This is the amount added. 3m*M*/l are used up in neutralising the amino acids, so the final concentration of bicarbonate is 30.5 m*M*/l.

vided. This solution, designated Medium *T*, consists of the first nine components listed in Table I at the concentrations there given. The modifications from Tyrode are that the NaCl is reduced to 0.61 per cent in order to make the final medium isotonic, the glucose is increased to 0.4 per cent and the $NaHCO_3$ to 0.28 per cent. The bicarbonate was increased so as to achieve a pH well on the alkaline side, which previous experience had shown to be beneficial. In this medium the lymphocytes survived fairly well for two days (Table II), but not so well for four days (Table III), and the cell quality was of course poor.

Amino Acids (Medium TA)

The next step was to add to medium *T* all the amino acids listed in Table I, giving Medium *TA*. The relative proportions of the amino acids is a rough approximation to the proportions present in plasma proteins and the total amino acid concentration is about double that in plasma. The results obtained (Tables II and III) showed that the amino acids improved the lympho-

TABLE II

Two-day cultures.
Vitamin concentrations, in μM/l, are given in brackets; *p*ABA = *p*-aminobenzoic acid.

Exp.	Medium	Pyknotic count	Cell quality	No. of cultures
	(Normal lymph nodes *in vivo*)	1.04 ± 0.07	2.5	58
1	Rat serum + 200 mg % glucose	1.38 ± 0.10	4	24
2	,,	1.77 ± 0.15	5	32
3	,,	1.61 ± 0.15	4	28
4	,,	1.40 ± 0.10	4	27
5	*T*	3.12 ± 0.22	5	21
6	*TA*	2.19 ± 0.20	4	26
7	*TA* + Thiamine (1)	1.37 ± 0.13	4	25
8	*TA* + *p*ABA (1)	1.33 ± 0.09	2.5	31
9	,,	1.76 ± 0.21	2	15
10	*TA* + Thiamine (1) + *p*ABA (1)	1.69 ± 0.15	2	17
11	,,	1.70 ± 0.19	2	21
12	*TA* + Thiamine (10) + *p*ABA (50)	1.50 ± 0.32	1.5	17
13	,,	1.07 ± 0.13	1.5	18
14	,,	1.04 ± 0.11	2	18
15	,,	0.88 ± 0.10	1.5	20
16	*TA* + Thiamine (50) + *p*ABA (250)	1.14 ± 0.17	1.3	17

cyte survival, especially over 4 days, and slightly improved the cell quality. The amino acids were not tested individually so it is not known if they are all required.

Vitamins (Medium TACP)

Next, various vitamins were added both singly and in groups to medium *TA*. Only two proved to be beneficial, thiamine and *p*-aminobenzoic acid. Cocarboxylase (thiamine pyrophosphate) seemed to be slightly better than thiamine itself and was adopted for the final medium.

In 2-day experiments (Table II) addition of either thiamine or *p*-aminobenzoic acid in μM concentration improved the pyknotic count, but the effects were not additive (Exps. 7–11). The most striking feature however was that *p*-aminobenzoic acid greatly improved the cell quality. Increasing the concentrations to 10 μM thiamine and 50 μM *p*-aminobenzoic acid further improved both pyknotic count and cell quality (Exps. 12–15). Further increase to 50 μM thiamine and 250 μM *p*-aminobenzoic produced no additional effect (Exp. 16). It may be noted that, over a two-day period at any rate, the last two media gave better results than serum and indeed the cell quality was better than in a normal lymph node.

In 4-day experiments (Table III) the improvement in pyknotic count brought about by either thiamine or *p*-aminobenzoic acid at μM concentration was again apparent, and in this case the effects were definitely additive (Exps. 20–27). The improved cell quality which, in 2-day experiments, had been obtained with *p*-aminobenzoic acid at μM concentration, was, in 4-day experiments, only obtained when the concentration was raised to 10 μM (Exps. 36–40).

Cell differentiation (as described in the section on Methods) only occurred when both thiamine (or cocarboxylase) and *p*-aminobenzoic acid were present, and it increased as the concentrations of both these substances were raised. Starting from Exp. 32 in which the concentrations of cocarboxylase and *p*-aminobenzoic acid were each μM, increasing the cocarboxylase to 10 μM had no effect (Exp. 34) but increasing the *p*-aminobenzoic acid to 10 μM considerably improved both cell quality and differentiation (Exps. 36–40). Now, with the cocarboxylase μM and the *p*-aminobenzoic acid 10 μM, increasing the cocarboxylase to 10 μM or the *p*-amino-benzoic acid to 50 μM, separately, had no effect (Exps. 44, 45), but when both were increased together there was a distinct further improvement in both cell quality and differentiation (Exps. 46–49). Further increases to 50 μM cocarboxylase and 250 μM *p*-aminobenzoic acid did not result in any significant

TABLE III

Four-day cultures.

Vitamin concentrations, in $\mu M/l$, are given in brackets; pABA = *p*-aminobenzoic acid, *p*OBA = *p*-hydroxybenzoic acid, Cocarb = cocarboxylase.

Exp.	Medium	Pyknotic count	Cell quality	Cell differentiation	No. of cultures
17	Rat serum + 200 mg % glucose	1.62 ± 0.17	4	−	19
18	,,	1.38 ± 0.16	4	−	18
19	*T*	17.56 ± 2.41	5	−	19
20	*TA*	7.98 ± 0.62	4	−	17
21	*TA* + Thiamine (1)	3.77 ± 0.26	4	−	20
22	,,	3.33 ± 0.42	4	−	18
23	*TA* + *p*ABA (1)	5.14 ± 0.37	3.5	−	16
24	,,	4.10 ± 0.40	3.5	−	20
25	*TA* + Thiamine (1) + *p*ABA (1)	2.23 ± 0.27	4	+	21
26	,,	2.18 ± 0.16	3.5	+	18
27	,,	2.30 ± 0.22	4	+	21
28	*TA* + Thiamine (1) + *p*OBA (1)	1.98 ± 0.12	4	+	21
29	*TA* + Thiamine (1) + Folinic acid (1)	2.14 ± 0.16	3	+	20
30	,,	2.56 ± 0.23	4	+	19
31	*TA* + Thiamine (1) + Folic Ac. (1)	3.17 ± 0.30	5	−	22
32	*TA* + Cocarb (1) + *p*ABA (1)	2.18 ± 0.21	3	+	16
33	*TA* + Cocarb (1) + *p*OBA (1)	1.73 ± 0.17	3.5	+	19
34	*TA* + Cocarb (10) + *p*ABA (1)	1.94 ± 0.14	3.5	+	20
35	*TA* + Thiamine (10) + *p*ABA (1)	3.71 ± 0.43	3.5	+	16
36	*TA* + Cocarb (1) + *p*ABA (10)	2.31 ± 0.58	2	+ +	19
37	,,	1.58 ± 0.17	2.5	+ + +	19
38	,,	3.64 ± 0.63	1.5	+ + +	16
39	,,	3.58 ± 0.45	2	+ +	17
40	,,	1.86 ± 0.23	2	+ +	19
41	*TA* + Cocarb (1) + *p*OBA (10)	2.28 ± 0.21	2	+ +	17
42	,,	3.26 ± 0.48	2.5	+ +	15
43	,,	1.84 ± 0.25	2	+ +	21
44	*TA* + Cocarb (10) + *p*ABA (10)	1.86 ± 0.25	2	+ + +	19
45	*TA* + Cocarb (1) + *p*ABA (50)	2.24 ± 0.30	2	+ +	18
46	*TA* + Cocarb (10) + *p*ABA (50)	1.85 ± 0.26	1.3	+ + + +	18
47	,,	3.05 ± 0.49	1.5	+ + + +	18

Table III (Cont.).

Exp.	Medium	Pyknotic count	Cell quality	Cell differentiation	No. of cultures
48	*TA* + Cocarb (10) + *p*ABA (50)	3.15 ± 0.47	2	+ + +	18
49	,,	1.90 ± 0.15	1.5	+ + + +	20
50	*TA* + Cocarb (50) + *p*ABA (250) (= TACP) .	1.35 ± 0.16	1.5	+ + +	18
51	,,	1.85 ± 0.23	2	+ + + +	20
52	,,	2.31 ± 0.24	1.5	+ + + +	17
53	,,	2.14 ± 0.21	1.3	+ + + +	18
54	,,	1.76 ± 0.14	1.5	+ + + +	20
55	,,	2.59 ± 0.27	1.5	+ + + +	20
56	,,	2.56 ± 0.42	1.5	+ + + +	19
57	*TACPI*	1.91 ± 0.28	1.5	+ + + +	18
58	,,	1.68 ± 0.25	1.5	+ + + +	20
59	,,	1.92 ± 0.28	1.5	+ + +	18
60	,,	2.64 ± 0.27	1.3	+ + + +	20
61	*TACPI*, Mannose instead of glucose . . .	1.42 ± 0.29	2	+ + +	20
62	,,	1.90 ± 0.27	2	+ +	19
63	*TACPI*, Fructose instead of glucose . . .	12.34 ± 1.66	5	−	19

improvement (Exps. 50–56), but, since these concentrations were quite non-toxic, they were adopted for the final medium, which at this stage is designated *TACP*.

In this medium cultures survived reliably for four days and some were still healthy after eight days, though by this time the number of lymphocytes was somewhat reduced owing to the disappearance of pyknotic cells by autolysis and phagocytosis. Beyond eight days all the lymphocytes died rather abruptly but the reticulum cells still survived.

p-Aminobenzoic acid is a component part of the folic acid molecule. It is believed however that cells cannot utlise folic acid itself but only a derivative called folinic acid (Leuconostoc Citrovorum Factor). In conformity with these ideas it was found that *p*-aminobenzoic acid could be replaced by folinic acid (Exps. 29, 30), but not by folic acid (Exp. 31). It was also found that *p*-aminobenzoic acid could be replaced by *p*-hydroxybenzoic acid (Exps. 28, 33, 41–43).

The slight superiority of cocarboxylase over thiamine is seen by comparing the following Experiments:– 32 with 25–27, 33 with 28, 34 with 35.

The following vitamins were found to have no effect when added in the concentrations stated ($\mu M/l$) to medium *TA* or *TACP*:– pyridoxine (1),

pyridoxal phosphate (1), nicotinic acid (1), nicotinamide (1), riboflavin (0.3), pantothenic acid (1), biotin (1), cyanocobalamin (1 and 33), choline (100), inositol (100), ascorbic acid (300), vitamin A alcohol (1), α-tocopheryl phosphate (1).

Insulin (Medium TACPI)

Addition of insulin (1 unit/ml) to medium *TACP* did not significantly alter the results as judged by the three criteria so far used (Exps. 57–60). Nevertheless it did have a noticeable effect in improving the staining properties of the cells. All the cells present, including the reticulum cells, stained a little more darkly with heamalum both in the nucleus and the cytoplasm. This suggests that the cells contained more protein or nucleic acid or both and that insulin promoted the synthesis of these substances. It is known that insulin promotes the synthesis of both protein and nucleic acid in fibroblast cultures [9]. Insulin was therefore included in the final medium, which is designated *TACPI*.

Many other additions and alterations were made to the medium but none of them improved it and the rest of this paper is concerned with negative results which seem to deserve a brief record.

Purines and Pyrimidines

It is known that *p*-aminobenzoic acid is a growth factor for many bacteria, in which it is required for the synthesis of folinic acid, a substance which is concerned in some way with the synthesis of purines and pyrimidines. If the beneficial effect of *p*-aminobenzoic acid on the cultures is due to promotion of purine and pyrimidine synthesis it might be possible to obtain equally good results by adding purines and pyrimidines to the medium instead. Experiments on these lines however were inconclusive. Addition of a mixture of adenine, guanine, thymine, cytosine and uracil, each at 10 μM final concentration, to medium *TAC* produced an improvement in some experiments but not in others. Sometimes the pyknotic count was improved, sometimes the cell quality, and sometimes cell differentiation occurred, but the results were inconstant and the improvement was always less than that obtained with 10 μM *p*-aminobenzoic acid. Addition of the same mixture to a medium already containing 10 or 50 μM *p*-aminobenzoic acid certainly produced no improvement at all.

The following substances had no effect when added singly at 10 μM concentration to media *TAC* or *TACP*:– adenine, guanine, thymine, cytosine,

uracil, hypoxanthine, orotic acid, adenosine, adenylic acid, thymidine. At 1.00 μM some of these substances were toxic. Addition of pure DNA (0.1 or 0.01 per cent) to medium *TACP* also had no effect.

Peptones and Proteins

In the following experiments it was shown that the amino acid mixture in the synthetic medium could not be replaced by certain purified peptones and proteins which are commercially available. 'Bactopeptone', 'Neopeptone' or 'Proteosepeptone' (Difco) in 0.1 per cent solution in medium *T* gave no better results than *T* alone and were clearly inferior to medium *TA*. The following proteins were tried, also in 0.1 per cent concentration: - gelatin (Difco), 'crystallised bovine plasma albumin' (Armour), 'bovine plasma gamma globulin' (Armour). In 2-day experiments (Table IV) both gelatin and albumin slightly improved medium *T*, but the globulin did not. In 4-day experiments on the other hand the gelatin was highly toxic and the toxic effect was equally marked whether it was added to medium *T*, *TA* or *TACP* (Exps. 67–71). It was only toxic to the lymphocytes, the reticulum cells

TABLE IV

Effects of adding proteins in 0.1 per cent concentration to various media.

Culture period	Exp.	Medium	Pyknotic count	Cell quality	No. of cultures
2 days	5	*T*	3.12±0.22	5	21
	64	*T* + Gelatin	2.51±0.20	3	16
	65	*T* + Albumin	2.38±0.42	3.5	17
	66	*T* + Globulin	5.43±0.63	4	19
4 days	19	*T*	17.56±2.41	5	19
	67	*T* + Gelatin + tryptophane (0.1 m*M*)	46.79±3.92	5	19
	68	„	55.54±4.48	5	18
	20	*TA*	7.98±0.62	4	17
	69	*TA* + Gelatin	13.95±2.52	4	18
	70	„	19.92±2.39	3	17
	52	*TACP*	2.31±0.24	1.5	17
	71	*TACP* + Gelatin	7.98±0.99	3	18
	72	*TACP* + Albumin	2.20±0.23	2	17
	73	*TCP* + Albumin	13.35±1.86	3.5	19
	74	„	15.84±0.83	4	19

remained quite healthy. Gelatin is deficient in tryptophane, but addition of tryptophane did not improve the results (Exps. 67, 68). This is the only case encountered of a substance being beneficial over the first 2 days but toxic after 4 days. Albumin was still non-toxic after 4 days but it did not improve medium *TACP* (Exp. 72) nor could it replace the amino acid mixture of *TACP* (Exps. 73, 74).

CO_2 and Bicarbonate

In the following experiments the culture chambers were gassed with pure oxygen and they contained a piece of NaOH-moistened filter paper to absorb the respiratory CO_2. Under these conditions of course the medium *TACP* became highly alkaline and about 20 per cent of the cells were dead after 2 days. The pH was therefore readjusted by reducing the $NaHCO_3$ concentration, and extra NaCl was added to maintain the original osmotic strength of the medium. When the $NaHCO_3$ was reduced to two-thirds of the original concentration, quite good results were obtained, despite the fact that the pH (9.6) was still much above normal (Table V. Exp. 76). But when the $NaHCO_3$ was reduced to one-fifth the results were worse, though the pH (8.2) was now nearly normal (Exps. 77, 78). Similarly in 4-day experiments it was found that reducing the $NaHCO_3$ was harmful (Exp. 79) and when the $NaHCO_3$ was entirely replaced by a weak phosphate buffer at normal pH the results were also poor (Exp. 80). The bicarbonate concentrations quoted refer to the amount originally added; under the conditions of the experiment much of it turned to carbonate.

Table V

Effects of reducing the concentrations of CO_2 and $NaHCO_3$ in medium *TACP*.

Culture period	Exp.	CO_2	$NaHCO_3$ (m*M*/l)	pH	Pyknotic count	Cell quality	Cell differentiation	No. of cultures
2 days	75	3 %	33.5	7.8	1.23 ± 0.13	1.5		17
	76	0	22.4	9.6	2.54 ± 0.22	2.5		20
	77	0	7	8.2	3.12 ± 0.30	4		16
	78	0	7	8.2	3.89 ± 0.68	4		18
4 days	52	3 %	33.5	7.8	2.31 ± 0.24	1.5	+ + + +	17
	79	0	13.4	9.0	3.27 ± 0.26	4	–	18
	80	0	0, + 5m*M* Na_2HPO_4	8.0	3.21 ± 0.47	3.5	–	20

These results show that bicarbonate (or carbonate) is an important constituent of the synthetic medium; in its absence cell quality greatly deteriorates and cell differentiation fails to occur. CO_2 and pH seemed to be of little importance, confirming conclusions reached in earlier experiments with serum medium [13].

Miscellaneous

It was shown that the glucose in medium *TACPI* could be replaced by mannose (Table III, Exps. 61, 62) but not by fructose (Exp. 63). The following substances added to *TACP* in the concentrations stated had no effect:– sodium formate (m*M*), sodium acetate (m*M*, 5 m*M*), taurine (m*M*), glutamine (m*M*), NH_4Cl (m*M*), creatine (m*M*, 10 m*M*), phosphocreatine (0.2, 0.5 m*M*), xanthopterin (10 μM) leucopterin (μM), coenzyme A (0.1 Lipmann unit/ml), thyroxin (μM). The following substances added to *TACP* were slightly toxic:– cysteine (2 m*M*), sodium oleate (0.1 m*M*), cholesterol (0.1 m*M*), $MnSO_4$ (10 μM).

Mitosis

In the normal lymph nodes *in vivo* about 1 per cent of the medium and large lymphocytes were found to be in mitosis. Mitosis was not seen in small lymphocytes and rarely in reticulum cells. In cultures maintained for 4 days in medium *TACP* mitoses were occasionally seen, but these only amounted to 0.2 per cent or less of the medium and large lymphocytes, which is almost negligible.

As already described, many substances were added to *TACP* without improving the results. On the three criteria so far used, there was perhaps not much room for improvement, but we were chiefly looking for some increase in mitosis and none was found. The next objective in the development of the synthetic medium must be to stimulate mitosis in the medium and large lymphocytes. Perhaps there is some specific stimulant—a lymphopoietic factor—waiting to be discovered, or it may simply be that mitosis awaits a certain combination of favourable circumstances which may prove very difficult to reproduce *in vitro*.

DISCUSSION

Synthetic media containing Tyrode constituents, all the amino acids and most of the vitamins were first introduced by White [15] and by Morgan,

Morton and Parker [8] (Mixture 199). According to the authors, embryonic muscle cells survived in these media for several weeks, but there was no growth. On the other hand, Earle and co-workers [3] reported that when fibroblasts were cultured in either of these media a large proportion of the cells were dead in 6 days. More recently, Healy, Fisher and Parker [4] have considerably improved upon Mixture 199 by increasing the concentrations of cysteine, glutathione and ascorbic acid and adding CoA, DPN and TPN. In this medium (No. 703) it is claimed that fibroblasts will not only survive but also multiply for several months. We have tested nearly all the sixty odd substances which Parker and his colleagues have used in their various media and found that, apart from Tyrode constituents, only amino acids, thiamine and *p*-aminobenzoic acid seemed to benefit lymph nodes *in vitro*.

Our results are more in keeping with those of Wolff, Haffen, Kieny and Wolff [16] who cultured various embryonic organs on the surface of an agar jelly containing synthetic nutrients. They found that the tibia, syrinx, testis and ovary of the chick or duck embryo would grow, differentiate and survive for about 7 days in a medium of Tyrode solution plus amino acids. It was also shown [5] that addition of *p*-aminobenzoic acid stimulated growth of the tibia, but no striking results were obtained with the other B vitamins. In our experiments *p*-aminobenzoic acid improved the survival of lymphocytes and also stimulated them to grow larger and turn into monocytes. It is probable that *p*-aminobenzoic acid is used for the synthesis of folinic acid and it is of interest to note that lymphocytes apparently contain about 0.1 μg/ml of folinic acid [12] and that when most of the lymphocytes in the body are destroyed by X-radiation there is a concomitant increase in the urinary excretion of folic acid [10].

Lasfargues and de Léo [6] found that fibroblasts would survive for about a week in a medium containing only Tyrode solution and cysteine and that survival was further improved by addition of cocarboxylase, and also by CoA, ATP and DPN. In our experiments cocarboxylase (or thiamine) improved the survival of lymphocytes. It is known that lymphocytes (and other white cells) contain about 1 μg/ml of thiamine and also that they break down thiamine into the thiazole and pyrimidine moieties. In chromic leukaemia the capacity of the lymphocytes to break down thiamine is greatly reduced and their thiamine content is increased about threefold [1].

In general, all the results so far obtained with synthetic media, including those reported here, agree in showing that cells can survive, differentiate and sometimes multiply for a certain length of time in protein-free media and that the nutrients required are glucose, amino acids, vitamins and coenzymes.

SUMMARY

Lymph nodes from young rats were cultured in fluid media. The cultures were supported at the surface of the medium on a grid of tantalum wire gauze. Each vessel contained 15–20 cultures in 5.5 ml of medium and the gas phase was 3 per cent CO_2 in oxygen. In most experiments the lymph nodes were cultured for 4 days.

After culture, film preparations were made and the state of each culture was assessed on the following criteria:– (1) the percentage of dead lymphocytes, (2) the 'quality' of the lymphocytes—well nourished cells were bigger and more basophilic than starved ones, and (3) the amount of cell differentiation which had occurred.

Synthetic culture media were prepared by adding various nutrient chemicals to a modified Tyrode solution. The best medium discovered contains inorganic salts, glucose, 19 amino acids, cocarboxylase, *p*-aminobenzoic and insulin, together with chloramphenicol as antibiotic and phenol red as indicator. This medium is designated *TACPI*. In it cultures survived reliably for 4 days and some survived fairly well for 8 days.

After 4 days in *TACPI* the cultures contained about 2 per cent of dead lymphocytes, the lymphocytes looked bigger and better nourished than those in normal lymph nodes, and a certain amount of differentiation had occurred—many of the reticulum cells were turning into large lymphocytes and some of the small lymphocytes had turned into monocytes. By contrast, lymph nodes cultured for 4 days in rat serum contained about the same percentage of dead lymphocytes but the cells looked poorly nourished and there was no differentiation. Good quality lymphocytes and cell differentiation were only obtained when the medium contained both cocarboxylase and *p*-aminobenzoic acid, and the optimum concentrations of these substances were determined. Bicarbonate was also shown to be necessary.

Many other substances, including most of the vitamins, purines and pyrimidines, CoA, DNA, peptones and proteins were added to the medium but none improved it.

Occasional mitoses of medium and large lymphocytes were found in the cultures, but so far no mitotic stimulant has been discovered.

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CONTINUED STUDIES ABOUT THE FIXATION WITH OSMIUM TETROXIDE

ELECTRON STAINS IV.

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The interest that has been devoted to the interaction phenomena between fixative and object is as old as the use of fixation techniques themselves. Osmium tetroxide early became an important constituent of fixing fluids [12]. But because of interference with staining reactions its use was rather limited and consequently few studies concerning its mode of action on tissues were made and these did not exceed the character of preliminary orientation. Today an increasing importance for the field of electron microscopy puts this substance anew in the focus of interest [2].

The present paper deals with the uptake of osmium by different tissues during different times and with the extractability of tissue constituents after osmium fixation.

MATERIAL AND METHODS

Fresh liver, muscle, skin and fat tissue as well as tail tendons from adult female wistar strain rats were cut into small cubes of about two mm side length. The tendons were cut into pieces of five mm length. The tissues were then washed in 0.9 per cent saline three times each, after which procedure the washing fluids appeared fairly clear. Fixation was performed in 1 per cent osmium tetroxide solutions buffered with veronal to pH 7.2 [11] using individual containers for each fixation time. Samples were then taken out of the fixing fluids after 1, 4 and 48 hours of fixation, washed in a little saline and submerged in fresh saline for one hour. After that time the tissues were washed with distilled water and transferred to extraction thumbles. The washing-water was added to the saline of 1 hour submersion and the extracted nitrogen estimated by means of standard micro-Kjeldahl technique: Table I, columns 1a and 1b. In a second procedure the tissue cubes were extracted with water for 12 h in a soxleth apparatus. The extracts of this and the foregoing procedure contained no clearly detectable amounts of polysaccharides, sugars or fats. Therefore they were assumed to contain mainly peptides, proteins and inorganic salts. The nitrogen was then counted as an average protein containing 16.5 per cent N, of which the dry weight was calculated (Table I, columns 2a and 2b). Subsequently the extraction was continued 12 h with ethanol. The dry residues of the ethanol extractions consisted

Table I

Tissue	hrs of fixation	*1a* mg	*1b* %	*2a* mg	*2b* %	*3a* mg	*3b* %	*4a* mg	*4b* %	*5* mg	*6* mg	*7* mg	*8* mg	*9* mg	*10* mg
Liver	1	10.83	1.9	291.4	52.1	50.5	9.0	10.23	24.8	248.80	31.08	217.72	559.62	41.31	0.0738
	4	11.22	1.7	346.5	54.2	54.8	8.6	31.22	40.9	283.10	45.14	237.96	639.26	76.36	0.1194
	48	8.14	1.2	392.8	59.7	61.8	9.3	41.35	41.4	262.50	58.42	204.08	658.28	99.77	0.1516
Muscle	1	33.48	2.9	344.6	31.3	105.4	9.6	1.21	3.4	687.00	34.50	652.50	1102.50	35.71	0.0323
	4	40.00	3.2	453.0	38.0	75.2	6.3	9.02	6.5	710.40	45.57	664.83	1193.03	54.59	0.0458
	48	21.25	1.7	552.3	43.8	110.5	8.8	27.26	25.9	675.10	78.08	597.02	1259.82	105.34	0.0836
Skin	1	17.20	0.9	436.3	24.2	671.6	37.2	1.00	1.9	747.90	50.84	697.06	1804.96	51.84	0.0287
	4	26.88	1.5	240.1	13.7	683.3	39.1	1.12	1.6	895.20	70.49	824.71	1748.11	71.61	0.0410
	48	21.60	1.2	228.8	12.5	757.1	41.4	3.46	3.6	937.20	92.46	844.74	1830.64	95.92	0.0524
Tendon	1	0.30	0.2	20.7	15.6	6.4	4.8	0.14	1.4	115.20	9.76	105.44	132.54	9.90	0.0075
	4	3.40	1.7	36.6	18.7	18.9	9.7	0.20	1.5	153.40	13.39	140.01	195.51	13.59	0.0695
	48	0.00	0.0	67.3	41.1	13.4	8.2	0.56	2.3	106.40	23.52	82.88	163.58	24.08	0.1472
Fat	1	—	—	—	—	1499.4	97.0	—	—	83.50	36.66	46.84	1546.24	36.66	0.0237
	4	—	—	—	—	2362.6	96.5	—	—	177.10	90.89	86.21	2448.81	90.89	0.0371
	48	—	—	—	—	2097.8	95.8	—	—	168.10	76.32	91.78	2189.58	76.32	0.0349

Explanation of the columns in the Table.

1) 1 hrs extract in saline.
  a) Nitrogen as protein in mg.
  b) Values from (1 a) in % of total dry weight.
2) 12 hrs extract with water in soxleth.
  a) Nitrogene counted as protein in mg.
  b) Values from (2 a) in % of total dry weight.
3) 12 hrs extract with ethanol in soxleth.
  a) Extract dry weight (lipids) in mg.
  b) Values from (2 b) in % of total dry weight.
4) Osmium contents of water extract 2.
  a) In mg.
  b) Values from (4 a) in % total Os-uptake.
5) Dry weight of extracted tissue in mg.
6) Osmium contents of extracted tissue in mg.
7) Corrected dry weight of extracted tissue in mg.
8) Total dry weight of tissue in mg (2 a + 3 a + 7).
9) Total osmium uptake in mg.
10) Osmium uptake by tissue in mg per mg dry weight [9, 10].

mainly of a white, fatty, crystalline mass. These ethanol extracts are regarded to be composed mainly of lipids (Table I, columns 3a and 3b). After 48 h of fixation only the extracts from fat tissue and skin tissue had a brown appearance, indicating that some osmium had been extracted too. The extracted tissues were then transferred to small crucibles and dried at 105°C and weighed (Table I, column 5).

Both the water extracts and the extracted tissues were analysed for their osmium content. For this purpose the methods of osmium analysis described in the literature were unsuitable, partly because their limit of detection prohibited an application to the amounts of osmium concerned here and partly because a considerable uncertainty is caused by ill defined titration end points, etc. Thus a colorimetric method of analysis based on the colour reaction of osmium tetroxide with thiourea discovered by Chugaev [6] was developed. The sensitivity of the method is sufficient to allow an estimation of as little as 0.001 mg Os/ml. The method is described in detail elsewhere [3]. In short it involves an alkaline digestion of the sample and subsequent oxidation with nitrate. From the produced osmate, osmium tetroxide is distilled off by aid of a dichromate-sulfuric acid mixture into hydrochloric acid and thiourea.

Precipitation of osmium in the water extracts was effected by addition of ethanol and diluted sulfuric acid. The solutions were filtered after 12 h and the filter residues analysed for osmium according to the method briefly outlined above.

RESULTS AND DISCUSSION

From the data collected in connection with earlier studies of the reactions of osmium tetroxide and ruthenium tetroxide [2] it was suggested that different tissues have different rates as well as different amounts of osmium uptake per unit dry weight from osmium tetroxide fixing fluids. This was of interest because the amount of osmium in an electron microscopical preparation will greatly influence the contrast and the general appearance of the picture. From the morphological point of view some distinct progress has already been achieved attacking the question of fixation influence on picture formation and the general ability to get "well preserved tissue structures" (for references see: [2]). But little is known to what extent the osmium itself participates in the formation of the picture, i.e. by increased electron scattering.

Such considerations led to the question of what general factors, from the point of view of the object, may be responsible for the picture formation. The contrast in electron microscopical pictures of biological material may be regarded as depending on the following conditions which do not include the primary dependence of picture formation and contrast from electron optical conditions in a given instrumental arrangement. The instrumental arrangements will be constant for most commercial electron microscopes, the variable size of objective apertures excluded. Some effects on contrast

from focussing have been realized by electron microscopists and treated theoretically [13].

(1) Contrast as object in the electron beam versus a nonscattering or faint scattering surrounding, i.e. virus particles, bacteria, tissue fractions or tissue culture cells on the membrane of the supporting grid.

(2) Contrast inside the object: *Different thickness* of layer of the electron scattering object parts, i.e. in preparations as listed under (1). *Different densities* in certain structures: If the average density of a biological structure is assumed to a certain value (i.e. 1.3) a homogenous material distribution in an even layer would give a certain "absorption" value for this material. An increase above this value in some structures, e.g. an increased mass density, can only be imagined at the expense of density in other parts of the total organisation. In biological material low scattering areas will perhaps be liquid-filled vesicles (density ~ 1) or structures whose density is considerably below the average value. Such considerations become particularly important for thin sections where embedding material is left in place, filling spaces which have been occupied previously by water, thus introducing a scattering background of similar elementary composition and with a similar density value as for the tissue average [4].

(3) Contrast due to the atomic properties that influence electron scattering and which can be simplified to a quotient $K \frac{\delta \times Z^{2*}}{A}$ [17] compared with scattering quotients for C, O, H, N, the "normal" tissue components. Such elements may be P, S, Fe, Mg, Ca, etc.

(4) Contrast due to increased density of the original hydrated structure as a fixation effect or precipitation of a soluble protein onto the surrounding structures.

(5) Contrast due to differential action of the electron beam on different object parts with resulting variation in layer thickness and/or density. This point is presently under investigation [5].

(6) Contrast through the presence of more or less specifically reacting, externally introduced, metals with high scattering quotient values, so called electron stains.

Since in modern fixation techniques for electron microscopy metals, and particularly osmium, play important roles, a study concerned with this last point, 6, seemed to be indicated and was therefore made along the following questions:

---

* δ = Density, Z = Atomic number, A = Atomic weight, K = Instrumental constant. Formula considerably simplified, and without reference to the S_w values of Hall [9].

(a) How much osmium will be found in different tissues after certain fixation times?
(b) Is there a characteristic pattern of osmium uptake for each tissue with time?
(c) Is the fixation with osmium tetroxide a binding of tissue components resulting in decreased solubility or extractability?
(d) Are all lipids bound to the tissue by osmium tetroxide fixation?
(e) To what extent does the expected destruction of tissue components proceed with the time of fixation?

In Fig. 1 (see also Table I, column 10) the osmium uptake per unit dry weight is plotted against the time of fixation. Osmium per unit dry weight is the total amount of osmium that has been taken up after the indicated time of fixation and which remains in the tissue after extraction for one hour with 0.9 per cent saline. It is the sum of the osmium found in the water and ethanol extracted tissues and the osmium found in the water extract. The dry weight in this figure is the total dry weight and is derived by adding the dry weight of the extracted tissue (corrected for its osmium content) to a protein dry weight calculated from the micro-Kjeldahl nitrogen in the water extract plus the dry weight of the ethanol extract. (Table I, columns 2 a + 3 a + 7.)

With the exception of tendon tissue the uptake curves for different tissues are strikingly similar (Fig. 1). It may therefore be justified to suggest a general form of uptake curve for tissues (dotted line) reaching a saturation point between four and eight hours. Although the curves show a common trend,

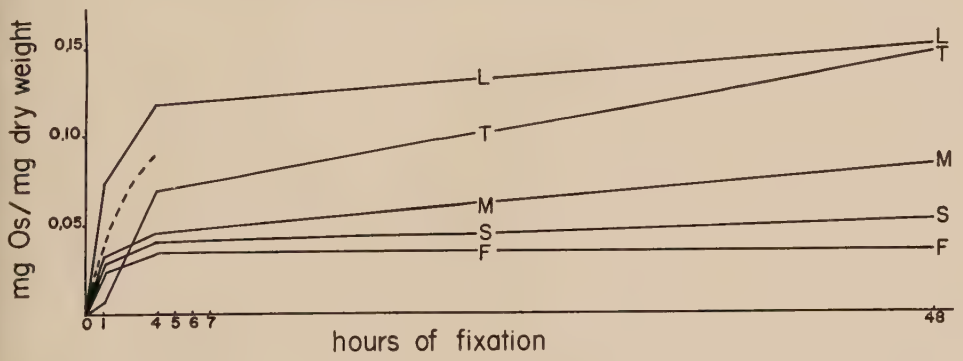

Fig. 1. demonstrates the osmium uptake of different tissues during different times. L = liver tissue, T = tendons, M = muscle tissue, S = skin tissue, F = fat tissue. Dotted line = Suggests a general form of uptake curve for tissues.

not only different uptake rates are demonstrated but it is also shown that there is a clear difference in intermediate and final osmium contents for the studied tissues. The slow rate of osmium uptake for tail tendons during the first hour of fixation may be caused by a particularly slow rate of penetration into the tightly packed collagen fibrils as well as a slow rate of chemical reaction with the substances themselves [2]. Quite puzzling however is the very high final uptake of osmium in tendons compared with other tissues. The low osmium values for fat tissues are understood when the high ethanol extraction figures for this tissue are regarded. Only a thin outer layer of every piece of fat tissue has reacted quickly with osmium tetroxide and prohibits a further penetration of the fixative. Fat tissue put into an osmium tetroxide fixation fluid is blackened immediately in agreement with the general behaviour of lipids containing double bonds. Therefore a comparison of the low fat value with values for other tissues would be misleading.

Differences in osmium contents encountered for liver, muscle and skin are significant and can be regarded as typical for the tissues in question. After 4 hours of fixation liver tissue contains more than two and a half times more osmium compared with muscle tissue and nearly three times as much as the skin sample for the same time of fixation. These differences in osmium content will no doubt greatly influence the final electron microscopical picture. Based on these facts and chemical data [2] a similar behaviour can be predicted for other parenchymatous tissues as pancreas and kidney.

Osmium uptake from fixing solutions of osmium tetroxide has been studied earlier by Palmer [16]. This author regarded osmium impregnation as complete when OsO_4 is no longer taken up by the tissue, as indicated by the concentration of the supernatant fluid becoming nearly stationary. This gives approximation to the optimum period of fixation. Palmer's study is the first attempt at a quantitative approach to the problem concerned here. Nevertheless it seems very doubtful that the methods used justify conclusions. A decrease in osmium tetroxide concentration in the fixing fluid is not inevitably caused solely by uptake of Os in the tissue. Palmer already assumed a rough proportionality between the amount of OsO_4 finally taken up and the volume of his piece of tissue. Varying uptake of osmium under different physiological conditions and by different tissues has been assumed in a few single observations [7, 11].

As to our knowledge no data about the extractability of substances other than lipids after osmium fixation are available in the literature. Fig. 2 is a

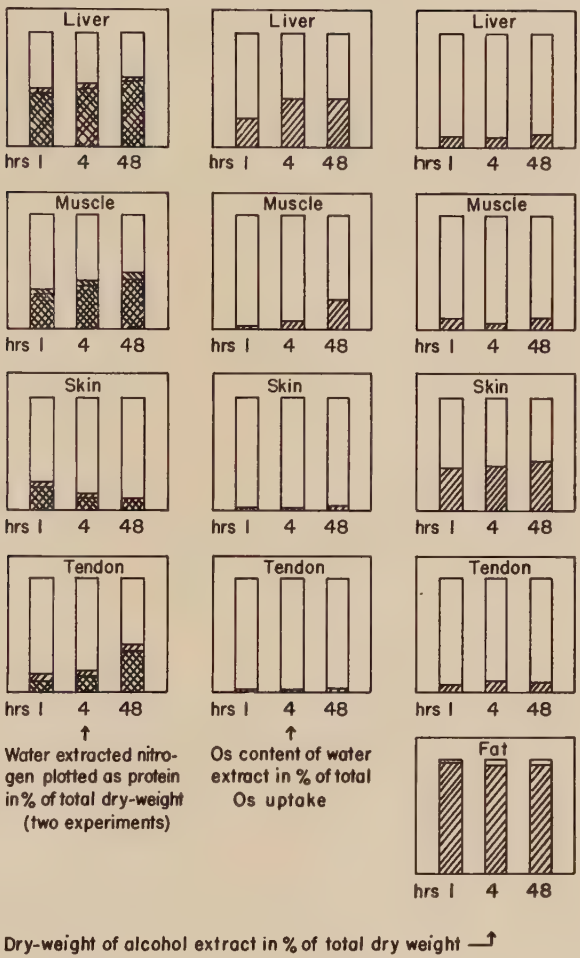
Liver
Muscle
Skin
Tendon
Fat
hrs 1 4 48
Water extracted nitrogen plotted as protein in % of total dry-weight (two experiments)
Os content of water extract in % of total Os uptake
Dry-weight of alcohol extract in % of total dry weight

Fig. 2. Extraction data from soxleth extractions after 1, 4, 48 hours of fixation in osmium tetroxide.

compilation of the more important extraction data collected during this study (see also Table I, columns 2 a–4 b). These data present at the same time a basis for judging the "fixing" properties of osmium tetroxide.

Regarding the histograms in Fig. 2 it has to be kept in mind that they are derived from very effective soxleth extractions during 12 h and will be high compared with normal preparation techniques for electron microscopy. An estimate of losses during extraction in saline for one hour can be obtained from extraction data. The extracted organic material calculated according to principles described above does not exceed 3.2 per cent (Table I, column 1 b). It therefore seems doubtful that a washing time of one to two hours

following a fixation time of four hours will serve any useful purpose when one considers the slow penetration rate of OsO_4—in other words, the rate of diffusion into and out of the tissue—and particularly since the rate will even be less because conditions have to be kept isotonic also during the washing procedure. On the other hand a "thorough" washing of the tissue will approach the figures for water extraction in Fig. 2, resulting in a considerable loss of tissue constituents.

The amount of extractable material is remarkably high. For liver it is in excess of 50 per cent. Liver, muscle and tendon exhibit a clear increase in extractability with time, thus indicating a progressing destruction by osmium tetroxide action. The sharp increase of extractability for tendon tissue that has been fixed for 48 h agrees well with the increase in osmium uptake for the same time (Fig. 1). The reverse effect on skin is not clear. Parallel to the extractability of organic matter osmium is extracted in increasing proportions.

Ethanol action results in rather constant amounts of extracted material for each tissue, although a marked decrease would be expected. The high values for fat tissue are explained by quick action of osmium tetroxide on the surface of the tissue pieces. The ethanol extract from tissues contains white fatty masses and does not show any signs of osmium blackening during the first 4 h of fixation. Only after 48 h of fixation a slight brown tone appears in the extract. The extracts still can be shown to contain unsaturated fatty acids. It is therefore possible that phenomena, similar to that for whole fat tissue, occur in micro scale in other tissues, i.e. a quick and insulating surface reaction of fat droplets with the penetrating osmium. This would explain some of the contradicting opinions about solubility of osmicated lipids [1, 10, 11, 14]. In addition to the above named observations the extractability of osmicated unsurated fatty acids in model experiments was studied. Linoleic, linolenic and arachidonic acid were mixed with stoichiometric amounts of OsO_4 in carbon tetrachloride, transferred to intestine tubes and put in ethanol. During 6 weeks no diffusion of fatty acid or osmium tetroxide into the ethanol occurred. A similar experiment with lecithin resulted in a slight diffusion, which is not regarded to be significant because of lack of stoichiometric conditions.

It can thus be said that the reaction products of unsaturated fatty acids with osmium tetroxide obviously are unable to pass through natural membranes. On the other hand it must be assumed that very tiny tissue pieces containing only small fat inclusions in their cytoplasm and fixed appropriate times will not suffer from considerable fat extraction effects.

SUMMARY

Fixation techniques using osmium tetroxide play a conspicuous part in electron microscopy of biological material. On account of its atomic electron scattering properties, the reduced osmium remaining in the tissue upon such treatment contributes significantly to the formation and contrast of the electron microscopical picture. Quantitative data pertaining to this problem are presented in this paper.

The osmium uptake from fixation fluids by different tissues reveals very similar and characteristic patterns. The rate of osmium uptake thereby varies for different tissues and the uptake is as a rule completed after 4 to 8 hours. The osmium content per unit dry weight (Fig. 1) varies in different tissue types.

In fat tissue the outer layers react very quickly with osmium tetroxide, thus preventing a further penetration of the fixation fluid into the tissue. From other tissues, constant amounts of fat are extractable, independent of the fixation time. It may thus be concluded that similar surface reactions occur with lipoid droplets in the the latter tissues.

Model experiments suggest that reaction products from unsaturated fatty acids and osmium tetroxide are unable to pass through natural membranes.

Osmium fixation, particularly when prolonged, brings about progressive destruction of tissue constituents and results in increased extractability with water.

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ÉTUDE AUTORADIOGRAPHIQUE DU MÉTABOLISME DES PROTÉINES ET DES ACIDES NUCLÉIQUES AU COURS DE L'OOGÉNÈSE CHEZ LES BATRACIENS

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Le métabolisme de l'oocyte en voie de croissance commence à être relativement bien connu : en particulier, la richesse en *acide ribonucléique* des jeunes oocytes a été mise en évidence tant par les méthodes cytochimiques que par les dosages chimiques. Chez les Batraciens, où l'apparition du vitellus tend à masquer progressivement la basophilie de l'hyaloplasme, une couronne basophile se maintient longuement au voisinage immédiat de la vésicule germinative [4, 22].

En ce qui concerne les protéines, Hultin [14] a montré, au moyen des radioisotopes, que la glycine et l'alanine, de même que l'ammonium, pénètrent rapidement dans les œufs vierges d'oursins. D'autre part, de nombreux auteurs [6, 16, 13, 15] ont étudié, par chromatographie, la composition en acides aminés des embryons d'Amphibiens au cours du développement. Brown, Callan et Leaf [5] ont même pu analyser par cette méthode la nature chimique du suc nucléaire chez *Xenopus* : ils y ont reconnu la présence de la glycine et de la phénylalanine, que nous avons utilisées comme précurseurs dans les expériences qui vont suivre. Chez la grenouille, la vésicule germinative contient de la dipeptidase [4, 8], les oocytes moyens étant relativement les plus riches en cet enzyme. Il en va sensiblement de même pour l'estérase, la ribonucléase et la phosphatase alcaline.

Les nucléoles demeurent, en général, avides de pyronine pendant toute l'oogénèse. Vincent [21] et surtout Baltus [1], qui ont travaillé sur des nucléoles isolés d'Astéries, ont attiré l'attention sur l'existence de divers enzymes dans cet organite, qui participe sans doute au métabolisme des acides nucléiques et des co-enzymes nucléotidiques.

Au cours d'un travail antérieur [9], nous avons suivi, par la méthode autoradiographique, l'incorporation de la glycine-1-C^{14} dans les oocytes d'*Asterias rubens* et nous avons pu ainsi mettre en évidence le rôle de premier plan joué par le nucléole dans l'activité synthétique de cette cellule.

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Il nous a paru intéressant de reprendre cette étude autoradiographique sur une espèce différente et de suivre l'évolution métabolique des protéines et des acides nucléiques au cours de l'oogénèse chez *Rana fusca*.

MATÉRIEL ET MÉTHODES

Une première expérience a été effectuée sur des femelles de *Rana fusca* tout au début de leur oogénèse; elle a eu pour but d'étudier comparativement l'incorporation *in vitro* et *in vivo* de la *glycine-1-C*^{14} dans les oocytes.

D'une part, des fragments d'ovaires ont été prélevés, lavés et placés pendant 5 heures dans de la solution d'Holtfreter stérile, additionnée de glycine-1-C^{14} à raison de 2 μC pour 5 cc de milieu.

D'autre part, 4 μC de glycine-1-C^{14}, en solution dans 0.5 cc de Ringer pour Batraciens, ont été injectés dans la cavité générale d'une grenouille rousse qui a été sacrifiée 48 heures plus tard.

Dans les deux cas, les fragments d'ovaires ont été lavés dans la solution physiologique additionnée de glycine non marquée, puis fixés par congélation-dissolution [17]; cette méthode de fixation nous avait donné des résultats cytologiques et autoradiographiques satisfaisants lors d'un travail antérieur [11].

Une série de préparations a été soumise à l'action de la ribonucléase pendant 2 heures à 37°, selon la méthode de Brachet [3], pour séparer la radioactivité des protéines de celle des acides nucléiques; on sait, en effet, que la glycine est un précurseur, à la fois des protéines et des acides nucléiques. L'activité des préparations traitées à la ribonucléase a été comparée à celle de coupes témoins qui avaient été traitées pendant le même temps par de l'eau distillée à 37°.

Les coupes de 10μ ont été recouvertes d'émulsion sensible aux électrons (G_5 Ilford in Gel form) et soumises au processus photographique suivant la technique décrite précédemment [10]. Après le lavage, les préparations ont été colorées au vert de méthyle-pyronine (méthode de Unna, un peu modifiée [10]).

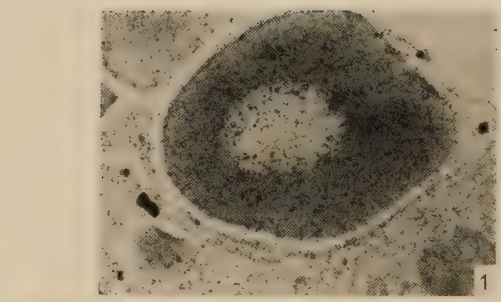
Une seconde expérience a permis de suivre la cinétique de l'incorporation *in vivo* de l'*adénine-8-*^{14}*C*: 3 animaux, qui se trouvaient respectivement au début, au milieu et à la fin de leur oogénèse, ont été injectés dans la cavité générale, d'adénine dont la radioactivité était de 4 μC. Ils ont été sacrifiés après 24 heures, 48 heures et 60 heures, respectivement. Dans ce cas encore, une série de coupes a été soumise à l'action de la ribonucléase.

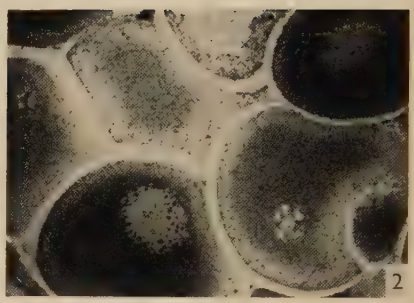
Lors d'une troisième expérience, une dose de 4 μC de *dl-3-phénylalanine-2-*^{14}*C*, en solution dans du Ringer pour Batraciens, a été administrée par injection à 2 femelles, qui se trouvaient l'une au milieu, l'autre à la fin de leur oogénèse. La première grenouille a été sacrifiée après 48 heures, la seconde après 60 heures.

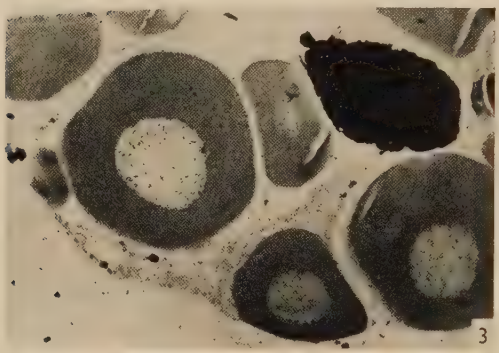
Une fois de plus, une série de coupes a été soumise à l'action de la ribonucléase dans le but de vérifier si la phénylalanine injectée constitue bien un précurseur spécifique des protéines.

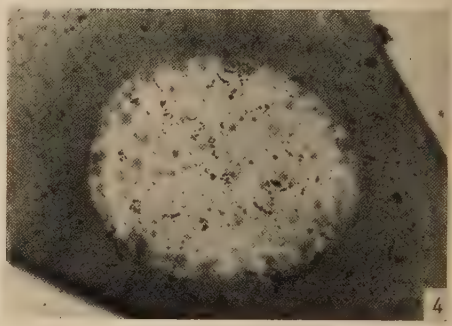
Dans toutes ces expériences, la fixation et l'autoradiographie ont été effectuées selon la technique qui a été indiquée plus haut.

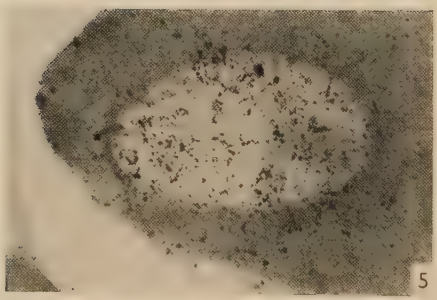
Lors des observations microscopiques, les oocytes ont été répartis en 3 classes: les petits oocytes, transparents, d'un diamètre de 20μ à 150μ; les oocytes moyens,











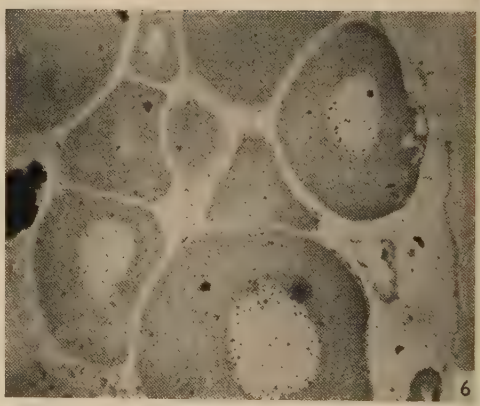
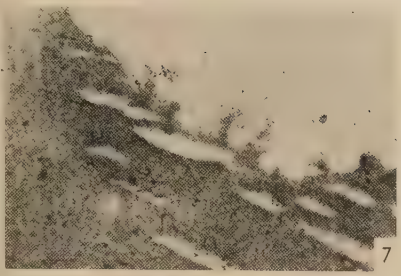
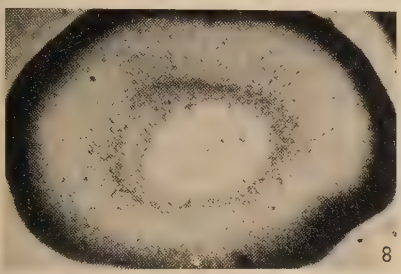
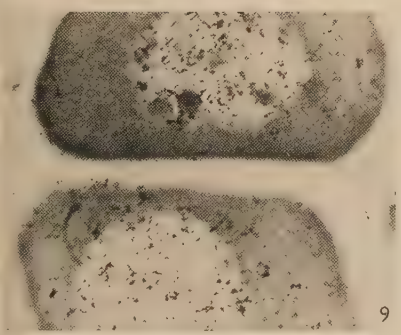
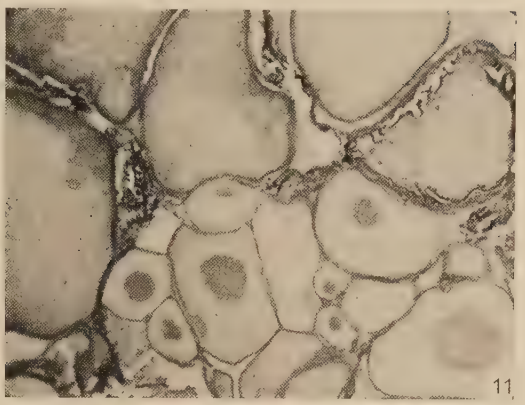


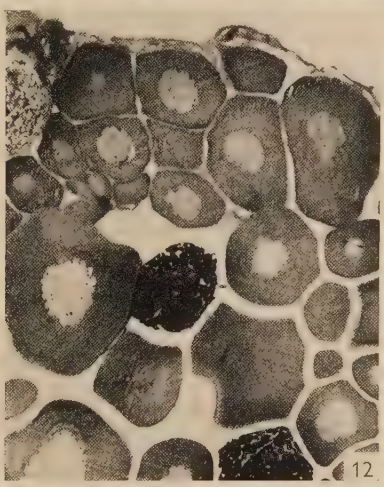
Fig. 1. Répartition de la glycine-1-C_{14} dans un oocyte de *Rana fusca* après un traitement de 5 heures *in vitro*. (2 jours d'exposition.)
Fig. 2. Coupe d'ovaire de *Rana fusca* après injection de glycine-1-C_{14}. Répartition homogène de la radioactivité.
Fig. 3. Ovaire traité *in vivo* par l'adénine-8-C_{14}. Après 24 heures, la radioactivité se localise dans la vésicule germinative. (3 jours d'exposition.)
Fig. 4. *id.* Détail. (2 jours d'exposition.)
Fig. 5. Oocyte traité *in vivo* par l'adénine-8-C_{14}. Après 60 heures, la radioactivité diffuse dans le cytoplasme. (4 jours d'exposition.)
Fig. 6. Action de la ribonucléase sur une coupe d'ovaire marqué par l'adénine pendant 24 heures. La radioactivité est complètement éliminée. (2 jours d'exposition.)











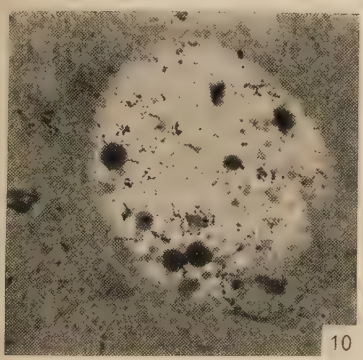


Fig. 7. Action de la ribonucléase sur une coupe d'oocyte traité pendant 60 heures par l'adénine-8-C_{14}. Quelques traces d'électrons subsistent dans la région périnucléaire basophile. (2 jours d'exposition.)
Fig. 8. Coupe d'oocyte en vitellogénèse, marqué par de la dl-phénylalanine-3-C_{14}. Un anneau radioactif est visible à la zone de contact entre le vitellus et la région basophile périnucléaire. (4 jours d'exposition.)
Fig. 9 et 10. Localisation de la radioactivité dans les nucléoles après le traitement par l'adénine-8-C_{14}. (3 jours d'exposition.)
Fig. 11. Coupe d'ovaire de *Rana fusca*. Coloration des protéines par le Bleu de Bromophénol. La vésicule germinative est plus intensément colorée que le cytoplasme.
Fig. 12. *id.* après l'action de la ribonucléase sur la coupe.

très basophiles, d'un diamètre de 150μ à 350μ et les gros oocytes, se trouvant au début ou au milieu de la vitellogénèse, d'un diamètre de 350μ à 900μ. Nous avons étudié séparément l'activité du cytoplasme et celle du noyau pour ces différentes classes d'oocytes. Une vingtaine de cellules de chaque type ont été observées dans chaque cas. Les valeurs qu'on trouvera plus loin au tableau I représentent le nombre de traces des électrons par unité de surface. La radioactivité a été calculée pour une durée uniforme d'exposition de 48 heures, temps adopté pour la majorité des préparations. Le back-ground cosmique étant négligeable pour ces durées brèves d'exposition (2 traces environ pour un champ microscopique de 1 mm^2, 538), il n'en a pas été tenu compte.

Nous avons effectué, en outre, quelques contrôles cytochimiques : outre la méthode de Unna, pour les acides nucléiques, la coloration des protéines par le bleu de bromophénol selon Mazia [18] et celle des protéines basiques par le jaune de naphtol [7] ont été utilisées dans ce but.

RÉSULTATS EXPÉRIMENTAUX

On trouvera dans le Tableau I, le résumé de nos observations.

Il ressort de ces observations que la radioactivité due à l'incorporation de la glycine se localise et se distribue de la même manière, qu'il s'agisse de l'expérience *in vivo* ou de celle *in vitro* (photos 1 et 2). Les valeurs, lors de l'administration *in vitro*, sont cependant plus élevées ; mais ce fait n'a probablement pas de signification importante puisque les doses administrées ont été choisies arbitrairement. Remarquons cependant que l'activité des cellules folliculeuses est beaucoup plus intense lors de l'incubation des ovaires *in vitro*. De toute façon, la radioactivité élevée de ces cellules, dans tous les cas étudiés, pourrait confirmer l'hypothèse souvent avancée [12] du rôle sécrétoire qui leur serait dévolu.

On notera que les oocytes moyens de 150 à 350μ de diamètre sont ceux où l'incorporation de la glycine est la plus considérable, tant dans la vésicule germinative que dans le cytoplasme. L'activité de la vésicule germinative est environ deux fois plus élevée que celle du cytoplasme, quel que soit le stade de l'oogénèse considéré.

L'action de la ribonucléase sur les coupes fait diminuer fortement la radioactivité, surtout celle des oocytes moyens très basophiles. Faisons toutefois remarquer que l'incubation pendant 2 heures à 37° dans de l'eau distillée suffit à extraire de 10 à 20 % de la radioactivité totale.

Dans le cas de l'*adénine*, les résultats les plus nets s'observent 24 heures après l'injection. On note, à ce moment, une localisation très nette de la radioactivité dans la vésicule germinative (photos 3 et 4). Après 48 heures, et plus encore après 60 heures, on constate que cette forte activité nucléaire se dilue dans le cytoplasme tandis que la radioactivité globale décroît (photo 5).

TABLEAU I

	Cytoplasme			Vésicule germinative		
Diamètre	Petits oocytes 20–150μ	Oocytes moyens 150–350μ	Gros oocytes 350–900μ	Petits oocytes	Oocytes moyens	Gros oocytes
Glycine-1-C^{14} *in vitro* (5 h)	3	8	2,2	6	13,6	5,8
Glycine-1-C^{14} *in vivo* (48 h)	2,2	6,6	2,2	8,4	11,6	6,8
Glycine *in vivo* + RNase	1	1,5	1,2	2	3,2	1,6
Adénine 1 (24 h)	0,3	0,3	0,2	4,9	9	4,2
Adénine 2 (48 h)	0,7	1,4	0,5	1	6	4
Adénine 3 (60 h)	0,7	2,3	0,2	0,7	2,5	3
Adénine 1 + RNase	0	0	0	0	0	0
Adénine 3 + RNase	0	0	0,07	0	0	0
Phénylalanine 2 (48 h)	4	3	2,8	4	8,5	4,4
Phénylalanine 3 (60 h)	1	1,7	1,3	1	3,5	1,8
(Glycine + RNase)	(1)	(1,5)	(1,2)	(2)	(3,2)	(1,6)
Phénylalanine 3 + RNase	–	1,4	0,8	–	3	1,3

Un traitement à la ribonucléase fait disparaître totalement la radioactivité des coupes, quand l'injection d'adénine précède de 24 heures la mort; on peut en déduire que la base purique s'incorpore exclusivement dans l'acide ribonucléique pendant ce laps de temps (photo 6). Mais, chez l'animal tué 60 heures après l'injection, la radioactivité du cytoplasme, surtout dans l'anneau périnucléaire, résiste à l'action de la ribonucléase sur les coupes : il est vraisemblable qu'à ce moment l'adénine s'est dégradée et qu'elle a cédé son carbone marqué aux protéines (photo 7).

Passons à l'incorporation de la *phénylalanine* : le maximum d'activité se manifeste 48 heures après l'injection. Après 60 heures, l'activité diminue, dans une même proportion, tant dans le cytoplasme que dans la vésicule germinative. Les oocytes où la vitellogénèse est en cours et où une zône périnucléaire basophile persiste encore présentent une concentration notable de traces à l'interface du vitellus et de l'anneau pyroninophile (photo 8). Il est intéressant de noter que la radioactivité des coupes pratiquées dans les ovaires traités par la phénylalanine pendant 60 heures est presque identique à celle des oocytes placés dans la glycine, puis soumis à la ribonucléase : l'activité résiduelle des protéines, dans ce dernier cas, est presque identique à celle trouvée dans le cas de la phénylalanine. Il faut en déduire que cette dernière constitue bien un précurseur spécifique des protéines; cette conclu-

sion se voit renforcée du fait qu'un traitement par la ribonucléase, dans le cas de la phénylalanine, n'abaisse l'activité que de 10 à 20 %, tout comme un simple séjour dans de l'eau distillée pendant 2 heures à 37°.

Quel que soit le précurseur utilisé, on observe toujours une certaine activité dans les noyaux vitellins. Quant aux oocytes en voie de dégénérescence, très colorés par la pyronine, ils sont presque inactifs; par contre, les cellules qui les phagocytent présentent une concentration de traces remarquable.

En général, ce sont les cellules les plus basophiles qui montrent le métabolisme protéique le plus intense dans leur cytoplasme et surtout dans leur noyau : dans les tout jeunes oocytes, encore transparents, l'incorporation des acides aminés dans les protéines du cytoplasme et dans celles de la vésicule germinative est du même ordre de grandeur; au contraire, dans les oocytes moyens très basophiles, dont le métabolisme protéique est beaucoup plus intense, la vésicule germinative présente une activité très élevée; dans tous les cas, cette activité est diffuse dans le suc nucléaire.

Plus tard, au cours du grand accroissement de l'oocyte et quand la vitellogénèse a débuté, la radioactivité nucléaire se localise essentiellement dans les nucléoles (photos 9 et 10).

Les préparations traitées par le jaune de naphtol ont donné des résultats plus reproductibles que celles qui ont été colorées au bleu de bromophénol; dans les deux cas, les nucléoles et les plaquettes vitellines prennent le colorant avec une intensité particulière. Il est intéressant de noter que la technique au bleu de bromophénol de Mazia nous a donné des résultats très différents selon que les coupes étaient traitées ou non à la RNase : comme nous l'ont montré des mesures de la densité optique, un tel traitement augmente beaucoup la colorabilité du cytoplasme, sans affecter fortement celle de la vésicule germinative (photos 11 et 12). Cet effet ne se retrouve pas dans le cas de la coloration au jaune de naphtol, qui semble moins influencée par la présence ou l'absence d'ARN et paraît donc plus favorable à une détermination quantitative de la teneur en protéines des préparations microscopiques.

DISCUSSION

La grande taille du matériel utilisé permet une localisation aisée de l'activité par la méthode autoradiographique; mais, comme l'oocyte constitue une type de cellule assez particulier, il peut être dangereux de vouloir généraliser ces données. Malgré ces réserves, nos observations confirment, une fois de plus, l'existence de liens étroits entre la teneur en acide ribonucléique de la cellule et l'intensité de son métabolisme protéique [2].

On peut aussi conclure que le nucléole doit être un centre de synthèse ou de turn-over particulièrement actif : nos constatations nouvelles cadrent à cet égard avec celles que nous avons déjà faites, par la même méthode, sur l'oocyte d'Astérie, au moyen de glycine-1-^{14}C; elles concordent aussi avec les données de Taylor [20], qui a travaillé sur les cellules du caecum gastrique, au moyen de ^{32}P et de l'autoradiographie par noircissement.

Un autre point qui mérite d'être signalé est le rôle très important joué par les protéines nucléaires au moment où la cellule est en pleine activité synthétique. Nos résultats concordent d'ailleurs bien avec ceux que Smellie et ses collaborateurs [19] ont obtenu dans le cas des protéines nucléaires, en travaillant sur des homogénats de foie et avec ceux que nous avons obtenus, sur la même cellule hépatique, par la méthode autoradiographique [11].

RÉSUMÉ

Une étude autoradiographique a été entreprise, au moyen des émulsions nucléaires, afin de suivre le métabolisme des protéines et des acides nucléiques dans les oocytes de *Rana fusca*, à différents stades de leur croissance.

Les précurseurs utilisés ont été la glycine-1-^{14}C, la dl-3-phénylalanine-2-^{14}C et l'adénine-8-^{14}C.

Les oocytes moyens, très basophiles, présentent une radioactivité particulièrement élevée, notamment au niveau de la vésicule germinative et surtout des nucléoles.

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OSMOTIC PROPERTIES OF PROTOPLASTS OF *BACILLUS MEGATERIUM*

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It has recently been shown that it is possible to dissolve the cell wall of *Bacillus megaterium* with lysozyme without ensuing lysis of the bacterial protoplasm [10]. This can be achieved by adding a stabilising substance, e.g. sucrose or polyethylene glycol, to the bacterial suspension. The protoplasmic bodies obtained have been named "protoplasts".

The properties of the protoplasts have been described in two papers [10, 11]. These bodies show, *inter alia*, the same endogenous respiration as the whole cells and the rate of glucose oxidation is unchanged. Low molecular weight substances, absorbing in the ultraviolet spectral range and present in the cells, do not leak out of the protoplasts. It has lately been found that this is true also of inorganic phosphate. These facts strongly suggest that the internal organisation of the bacterial cell is not seriously damaged by the removal of the cell wall, even if growth and division of the protoplasts have not been observed so far.

Investigations of the permeability properties of bacteria are rendered difficult by the circumstance that the cells are not always plasmolysable. *B. megaterium* is not plasmolysable according to Fisher [2], as has been confirmed by the author. By applying another technique, the permeability properties of this bacterium have, however, recently been investigated [12]. In connection with this study it was considered desirable to investigate the permeability properties of the corresponding protoplasts (as defined above) as well. These properties have now been studied by means of osmotic pressure experiments. The results are given in this paper, together with a discussion of the implications in regard to the structure of the intact bacterial cell.

Osmotic pressure experiments have earlier been performed on cells of a great number of higher organisms, showing that a semipermeable membrane is present at or near the protoplasmic surface. Very exact measurements have been performed on sea urchin eggs by Lucké and McCutcheon [6]. These authors were able to show that the volume V of the eggs is related to the osmotic pressure P of the surrounding medium by the formula

$$P(V - b) = \text{const.} \quad (1)$$

where b is the volume of the osmoticly inert substances (proteins, lipids, high molecular weight carbohydrates, bound water) of the egg. It will be shown in this paper that the same formula also applies to the bacterial protoplasts under certain conditions.

When this paper had been completed the author came across a recent publication of H. Stähelin [9] describing the permeability properties of protoplasts of *Bacillus anthracis*. The experiments performed by him are partly similar to those described below. A comparison between the results suggest that the permeability properties of the two bacterial species are at least qualitatively the same.

MATERIALS AND METHODS

B. megaterium, strain KM [7] was used in all experiments. Cultures for experimental work were grown in 2 per cent Difco peptone at 30°C with mechanical agitation using Erlenmeyer or Fernbach flasks. The cells were harvested during the phase of declining growth rate (6–12 hour cultures).

The polyethylene glycol (*PEG*) used was "Polyglycol A 4000", having an average molecular weight of 3500, as determined by end group analysis. The compound was obtained from Mo och Domsjö AB, Örnsköldsvik, Sweden.

Optical equipment. An Ortholux microscope equipped with an apochromatic 90 × oil immersion lens and a Leitz ocular micrometer (12.5 × eyepiece) was used for measurements of the diameter of the spherical protoplasts. In each preparation 50–400 protoplasts were measured depending on the precision needed.

The dry weight of bacterial suspensions was determined after heating samples at 100°C to constant weight.

The number of protoplasts in a suspension was determined in a Bürker counting chamber (depth 0.1 mm). The protoplasts were fixed in 4 per cent formaldehyde and a suitably diluted sample of the suspension was introduced in the counting chamber. The protoplasts were allowed to settle for ten minutes and a photograph was taken at a low microscopical magnification. The protoplasts were then counted on the photographical plate. 8 diluted samples were prepared from each suspension and from each sample about 600 particles were counted. The standard deviation of the particle count obtained was within ± 3 per cent.

Osmotic pressure determinations (*relative values*). The osmotic pressure of sucrose solutions are given in [5]. The pressure is not exactly proportional to the concentration, but as far as this investigation is concerned, the deviations are insignificant in comparison to other sources of error.

The osmotic pressure of a solution of PEG is not easily determined directly on account of the comparatively low molecular weight of the solute. A good estimation of the osmotic pressure can, however, be obtained by determining the freezing point depression of the solution, since the latter quantity is proportional to the osmotic pressure or at least nearly so.

The freezing point of PEG solutions of various concentrations was determined in a Dewar vessel, containing known amounts of solution and ice. After temperature equilibrium had been reached (aided by vigorous stirring) the temperature was read with a Beckman thermometer. The amount of ice melting during the experiment was determined by weighing. In order to obtain the freezing point depressions for the PEG solutions the freezing point of pure water was determined in the same way.

EXPERIMENTAL RESULTS

As has been mentioned already, a stabilising substance must be added to the bacterial suspension before the cell wall is dissolved by lysozyme in order to protect the protoplasts from further lysis. The experimental findings suggest that the stabilisation is not a purely osmotic effect; for example, cellobiose is not effective as a stabiliser [10] whereas sucrose is. Phosphate buffers, too, do not prevent complete lysis in spite of the fact that the buffer ions do not penetrate into the protoplasm of the living cell under the conditions prevailing during the lysis experiments [12]. In the present preliminary investigation as simple systems as possible were wanted. Therefore, in most cases the desired variations of the osmotic pressure of the medium was simply changed by varying the concentration of the stabiliser.

a. *The osmotic pressure of the medium surrounding the bacterial protoplasts.*—The solutions surrounding the protoplasts essentially contained: phosphate buffer of pH 7.0, 0.001*M*; lysozyme, final concentration less than 0.1 per cent; material from the cell walls; sucrose, 0.125–0.500*M*, or PEG, 5.8–16 per cent (w/v). Of these substances, the buffer ions and lysozyme exert a very small influence on the osmotic pressure in comparison with the sucrose or PEG.

The material from the cell walls, brought into solution by the lysozyme treatment, has to the greater part a molecular weight of 10,000–20,000 [8]. No certain differences in the results could be found when osmotic experiments were conducted with dilute (4 mg bacterial dry weight/ml) or concentrated (40 mg/ml) bacterial suspensions. For this reason it was not considered necessary to separate the protoplasts from the cell wall material by centrifugations, especially as these bodies are rendered somewhat less stable by such a treatment.

As mentioned above, the osmotic pressure of sucrose solutions was taken as proportional to the concentration of the solute and that of PEG solutions to their freezing point depression. Fig. 1 and Table I show that the freezing point depression, and hence the osmotic pressure, of the PEG solutions is far greater than should be expected from the molar concentration. It can also

TABLE I

Δt_e = experimentally found freezing point depressions for PEG solutions of various concentrations. Δt_c = calculated depressions assuming an average molecular weight of 3500 for the PEG.

% PEG in solution	Δt_e	Δt_c	$\Delta t_e/\Delta t_c$
4.0	0.035	0.021	1.6
7.2	0.085	0.038	2.2
12.0	0.228	0.064	3.6
17.0	0.471	0.091	5.2

TABLE II a

Number and volume of protoplasts. The protoplasts were obtained by lysozyme treatment of *B. megaterium* in PEG solutions of various concentrations. Dry weight of the whole cells = 39.5 mg/ml suspension. The error limits represent standard deviations.

	% PEG in solution		
	5.8	7.7	16.0
Average vol. of one protoplast, ml	$(2.52 \pm 0.05) \times 10^{-12}$	$(2.52 \pm 0.05) \times 10^{-12}$	$(2.48 \pm 0.05) \times 10^{-12}$
Number of protoplasts/g dried whole cells . . .	$(5.37 \pm 0.09) \times 10^{11}$	$(5.27 \pm 0.11) \times 10^{11}$	$(5.52 \pm 0.12) \times 10^{11}$
Total volume of protoplasts, ml/g dried whole cells	1.35 ± 0.04	1.33 ± 0.04	1.37 ± 0.04

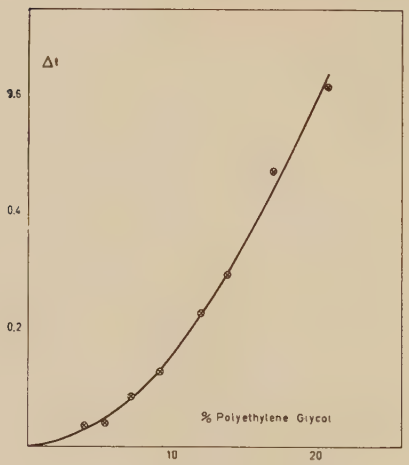
TABLE II b

Number and volume of protoplasts. The protoplasts were obtained by lysozyme treatment of *B. megaterium* in sucrose solutions of various concentrations. Dry weight of the whole cells = 34.7 mg/ml suspension. The error limits represent standard deviations.

	Sucrose concentration		
	0.125	0.200	0.400
Average vol. of one protoplast, ml	$(2.49 \pm 0.05) \times 10^{-12}$	$(2.32 \pm 0.03) \times 10^{-12}$	$(2.19 \pm 0.05) \times 10^{-12}$
Number of protoplasts/ dried whole cells . . .	$(4.64 \pm 0.10) \times 10^{11}$	$(4.75 \pm 0.10) \times 10^{11}$	$(5.42 \pm 0.08) \times 10^{11}$
Total volume of protoplasts, ml/g dried whole cells	1.13 ± 0.04	1.10 ± 0.04	1.18 ± 0.03

be seen that, in this case, the osmotic pressure does not increase proportionally to the concentration of the solute.

b. *The volume of protoplasts originating from bacterial suspensions containing varying amounts of PEG or sucrose.*—To 2 ml of a bacterial suspension of known dry weight content a concentrated solution of PEG or sucrose was added to the desired final concentration. The dissolution of the cell wall



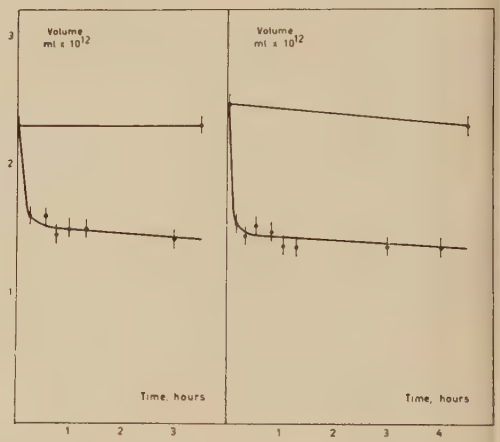


Fig. 1 (left). Freezing point depression, Δt, of PEG solutions.
Fig. 2 (right). Plasmolysis of bacterial protoplasts as a function of time. To the left: experiment in PEG solutions. Initial concentration: 5.8 per cent. At time 0 the concentration was increased to 16 per cent. To the right: experiment in sucrose. Initial concentration 0.125M. At time 0 the concentration was increased to 0.5M.

was effected by 0.1–0.2 ml of 1 per cent lysozyme. The experiments were performed in narrow and closed test tubes on account of the instability of the protoplasts in the presence of air [8]. The diameter of 400 protoplasts was measured. Tables II a and II b give the average values of the volume of one protoplast, the number of protoplasts/g dried whole cells and the total volume of the protoplasts in PEG or sucrose solutions of various concentrations. The standard deviation of the product (volume of one protoplast) × (number of protoplasts) = (total volume of protoplasts) was calculated according to the formula given by Dahlberg [1].

As can be seen from Table II a, the volume and number of protoplasts in PEG solutions is independent of the concentration of the solute.

In sucrose the volume of one protoplast slightly decreases with the sucrose concentration, but this decrease is balanced by an increase in the number

of protoplasts. The total volume of the protoplasts is thus practically independent of the sucrose concentration.

The total average volume of the protoplasts per g of dried whole cells is 1.34 ± 0.05 ml in PEG solutions. The corresponding value for sucrose is 1.14 ± 0.04. The difference is statistically significant.

c. *Volume changes of the protoplasts effected by changes in the concentration of stabiliser after removal of the cell wall.*—The lysozyme treatment was performed as described in section b above. The initial concentration of stabiliser was $0.125M$ (sucrose) or 5.8 per cent (PEG). Lower concentrations of the stabiliser do not confer satisfactory stability to the protoplasts.

Once the protoplasts had been formed additional amounts of sucrose or PEG were added in order to increase the osmotic pressure of the medium. It can be seen from Fig. 2 that considerable and statistically significant changes of the protoplast volume then occur. The average volume of one protoplast thus rapidly diminishes but soon assumes an almost stable value. Counts of the protoplasts before and after the experiments revealed no statistically significant changes in the number of protoplasts.

Plasmolysis was also tried by adding other solutes than PEG or sucrose to a suspension of protoplasts. Glucose caused a plasmolysis similar to that effected by sucrose. A decrease of the protoplast volume was observed when concentrated phosphate buffer, pH 7.0, was added, but at the same time the greater part of the protoplasts disintegrated. Urethan caused no plasmolysis.

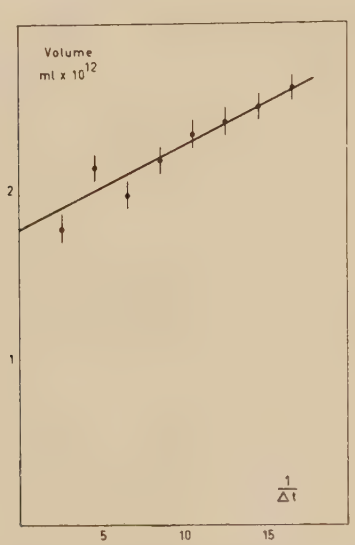
Some experiments were performed in order to study the reversibility of the plasmolysis. In the case of PEG solutions protoplasts were prepared in a medium containing 5.8 per cent of the stabiliser. The average volume of the protoplasts was determined and PEG was added to a final concentration of

TABLE III

Plasmolysis of protoplasts in PEG solutions. The average volume of the protoplasts was determined after each addition of PEG or water. For further data, see the text.

Addition of	Average vol. of protoplasts, ml $\times 10^{12}$
	2.30 ± 0.08 (initial vol.)
PEG	1.59 ± 0.06
Water	2.36 ± 0.08
PEG	1.48 ± 0.06
Water	2.48 ± 0.08
PEG	1.36 ± 0.06

16 per cent. After a new volume determination the initial concentration of PEG was restored by additon of 0.001M phosphate. The cycle was then repeated. Table III shows that the plasmolysis is nearly reversible a number of times. It should, however, be mentioned that a considerable amount of protoplasts disintegrate during experiments of this type. This is still more apparent in the case of sucrose solutions. A reversible plasmolysis can be



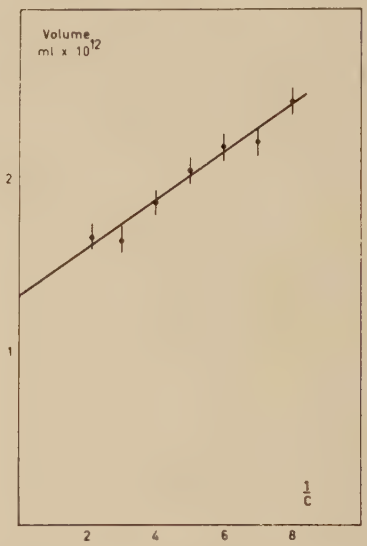


Fig. 3 (left). Plasmolysis of protoplasts in Peg solutions as a function of the osmotic pressure of the medium. The volume of the plasmolysed protoplasts is plotted against the reciprocal value of the corresponding freezing point depression of the solution. The straight line is drawn by the method of least squares.

Fig. 4 (right). Plasmolysis of protoplasts in sucrose as a function of the osmotic pressure of the medium. The volume of the plasmolysed protoplasts is plotted against the reciprocal value of the corresponding sucrose concentration (M/l). The straight line is drawn by the method of least squares.

observed even here, but the increased instability renders difficult accurate measurements of the volume changes.

In order to test the validity of formula (1) in regard to bacterial protoplasts, suspensions containing 5.8 per cent of PEG or 0.125M sucrose were prepared. Additional amounts of stabiliser were then added and the dependence of the protoplast volume on the osmotic pressure of the medium was determined. The results are seen in Figs. 3 and 4. The average volume of one protoplast, V, is plotted against $1/P$ (as represented by $1/c$ and $1/\Delta t$, respectively). Since formula (1) can be written in the form

$$V = \text{const.}/P + b$$

the linear relationship prevailing between V and $1/P$ (see Figs. 3 and 4) shows that formula (1) is valid, at least approximately, at the osmotic pressures applied. At still higher pressures the volume decreases more than is required by the formula, but at the same time the protoplasts show signs of deformation.

For $1/P = 0$ we get $V = b =$ the volume of the osmoticly inert substances of the protoplast. Fig. 3 gives a value of $b = 1.77 \times 10^{-12}$ ml $= 68$ per cent of the protoplast volume before plasmolysis. The corresponding values obtained with sucrose solutions (Fig. 4) are 1.33×10^{-12} ml and 54 per cent.

d. *Determinations of the dry weight of the protoplasts in terms of the dry weight of whole cells.*—It was considered desirable to correlate the volume of the osmoticly inert substances of the protoplasts (see section c above) with the volume and weight of the desiccated material present in the same bodies. The weight of the cell wall has earlier been determined by an indirect method [10]. The same quantity was now obtained in a more direct way by gravimetric analyses of whole cells and protoplasts. A bacterial suspension of known dry weight content was prepared. Sucrose was added to a final concentration of 0.2*M*. Protoplasts were then prepared by treatment with lysozyme and fixed with 4 per cent formaldehyde. The suspension was washed three times by centrifugations and the dry weight of the protoplasts was determined. A parallel experiment was performed with whole cells excluding the lysozyme. The percentage of protoplasmic and wall material in the cells was calculated from the dry weights of protoplasts and whole cells. According to the data given in Table IV 62 per cent of the total cell weight represent protoplasmic material and consequently 38 per cent cell material (dry weights).

TABLE IV

Determination of the dry weight of protoplasts in terms of the dry weight of whole cells. Duplicate samples of protoplasts and cells were fixed in 4 per cent formaldehyde, washed by centrifuging and weighed after heating at 100°C.

	Dry weight of protoplasts	Dry weight of whole cells	% protoplasmic material in cells
I	40.9	64.1	63.8
II	51.5	84.9	60.6
			Average 62.2

DISCUSSION

The experiments described in section b above show that the osmotic pressure of the medium before the cell wall is removed from the bacterial cells has little or no influence on the volume of the protoplasts. Consequently, the experimental data given in this paper do not allow an estimation of the osmotic pressure inside the intact bacterial cell.

Once the protoplasts are formed, however, they respond to changes in the osmotic pressure of the external medium (section c above). Plasmolysis experiments show that the volume decreases when the pressure is increased and vice versa. The volume changes obey the modified Boyle-Van't Hoff law $P(V-b)=\text{const.}$

The independence of the protoplast volume of the initial osmotic pressure of the medium could be explained by assuming a free passage of low molecular weight substances into and out of the protoplasts. The volume would then be more or less independent of the osmotic pressure of the medium. This explanation is, however, not very probable considering the fact that low molecular weight, ultraviolet absorbing substances and inorganic phosphate do not leak out of the protoplasts (cf. the introduction).

On account of analytical difficulties it has not been ascertained whether or not some of the sucrose or PEG penetrates into the protoplasts. The experiments described in section c above suggest that the protoplasts are impermeable to these substances once the protoplasts are formed: a plasmolysis instantaneously starts when the osmotic pressure is increased in the external medium. The plasmolysis, in addition, is reversible, in the case of PEG in a nearly quantitative way.

The presence of a permeability barrier at the surface of the protoplasm is most strongly indicated by the fact that the volume changes of the protoplasts follow the modified Boyle-Van't Hoff law. In the case the protoplasts represented membrane-less gel bodies these changes would not follow this law [4].

The average volume of the protoplasm in living, whole cells has earlier been determined to 2.53 ml per g of dried cells [12]. A comparison between this figure and the volume of the protoplasts, given in Table II show that the protoplasm shrinks to about half its original volume when the cell wall is removed.

As has been emphasized the volume of the protoplasts is independent of the osmotic pressure of the medium before the cell wall is dissolved. This indicates that the structure of the bacterial protoplasm is slightly changed when the free protoplasts [as defined in ref. 10] are formed. Hence the proto-

plast cannot be regarded as strictly equivalent to the protoplasm of the living cell, but some tentative conclusions concerning the structure of the living cell could nevertheless be justified. The validity of such conclusions is strengthened by the fact that the permeability of the living cell and that of the protoplast is qualitatively the same in regard to sucrose, phosphate and urethan [ref. 10 and section c above].

From the plasmolysis experiments illustrated by Figs. 3 and 4 an estimation of the osmoticly inert substances could be made (section c above). In PEG solutions a value of 68 per cent of the original protoplast volume (Table II b) = 0.91 ml per g of dried whole cells is obtained for this quantity. The volume of the protoplasm in the intact cells is approximately 2.53 ml per g cell weight [12]. The volume of the osmoticly inert substances accordingly represent $0.91 \times 100/2.53 = 36$ per cent of the protoplasm of the living cell. The remaining part of the protoplasm, 1.62 ml, should then represent the "free space" inside the cells, containing osmoticly transferable water and dissolved low molecular substances.

The quantity 0.91 ml may also be compared with the volume of the desiccated protoplasmic constituents. The indirect method for determination of the content of cell wall material in whole cells, outlined in ref. [10], gave a value of 20 per cent for the percentage of cell wall material in the cells (dry weights). Changes in the cell volume following the lysozyme treatment were, however, not taken into consideration. If the value of the protoplast volume given in this paper (Table II a, line 3) is used, a much higher value is obtained, namely 45 per cent. This value agrees reasonably well with the value of $100 - 62 = 38$ per cent, obtained from the experiments described in section d above (Table IV). The desiccated protoplasmic constituents can therefore, with a reasonably degree of accuracy, be assumed to represent 62 per cent of the total bacterial dry weight. If a partial specific volume of 0.70 is assumed [12] we get the volume of these substances = 0.43 ml per g of dried whole cells. The difference $0.91 - 0.43 = 0.48$ ml probably represents bound water. The presence of bound water has earlier been demonstrated by a similar method [3].

From the experiments with sucrose solutions a value of 0.62 ml is obtained for the volume of the osmoticly inert substances (Fig. 4). The corresponding amount of bound water, 0.19 ml, is considerably smaller than the amount calculated from the experiments with PEG solutions. Since the plasmolysis renders the protoplasts less stable in sucrose than in PEG solutions (see section c above) the experiments performed in the latter medium more likely reflect the conditions prevailing in the living cells.

SUMMARY

The osmotic properties of bacterial protoplasts have been investigated. The protoplasts were obtained by treating *Bacillus megaterium* cells suspended into polyethylene glycol or sucrose solutions with lysozyme.

The concentration of these substances and hence the osmotic pressure of the medium before the removal of the cell wall has little influence on the volume of the formed protoplasts. Changes in the osmotic pressure after the removal of the cell wall, on the contrary, causes plasmolysis of the protoplasts.

Determinations of the degree of plasmolysis as a function of the osmotic pressure indicate the presence of a permeability barrier against the external medium.

The performed measurements give information on the amounts of osmoticly inert substances and "free space" in the bacterial cell.

The author thanks Prof. A. Tiselius for his kind interest in this work, which was performed at his institute. The author is also very much indebted to Mrs. Karin Axén for skillful technical assistance.

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A METHOD FOR THE QUANTITATIVE ASSESSMENT OF AUTORADIOGRAPHS[1]

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The technique of high resolution autoradiography is capable of yielding information about events within single cells such as is obtainable by no other method. Granted the possibility of still further improvements in resolution, the chief limitation is the difficulty of quantitative assay.

An electronic grain counter based on the "flying spot" principle has been described by Dudley and Pelc [4]. Such a system is necessarily quite intricate, and details of its performance are not yet available. We describe below an optical system for scanning a strip of an autoradiograph, comparing the numbers of grains in any areas selected and registering quantitatively the profile of their distribution. The advantages as well as the sources of error will be considered. The method applies to "grain pattern" autographs made on thin film, not to 'track" autographs, whose merits and limitations have recently been discussed by Levi [5].

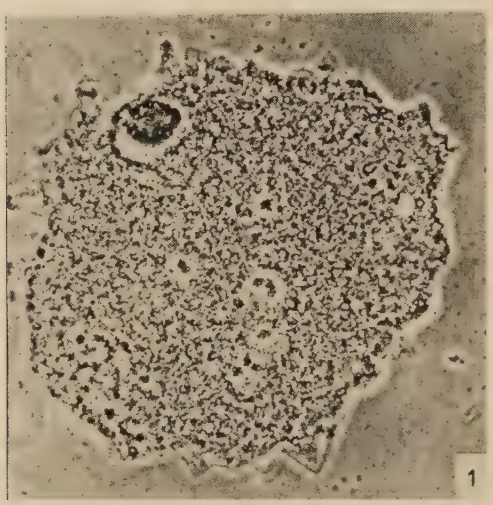
Principle of the Method

The distribution of grains along a long, narrow strip of the autoradiograph is measured in this procedure. The width of the strip will be determined by the experiment itself. For example, if a nucleus is to be compared with cytoplasm, the width of the strip will be adjusted to the width of the nucleus.

The system of observation must be such that each silver grain may be considered as a source of light. Let us assume that all grains are equal. The strip of the autoradiograph is observed through a cylindrical lens, its cylinder axis parallel to the long axis of the strip. This spreads each point of light into a band of light. If two grains lie in tandem along the strip, two bands

[1] This investigation was made possible by University of California Cancer Research funds and by grants from the American Cancer Society, recommended by the Committee on Growth, National Research Council. We are indebted to Professor J. E. Gullberg for valuable advice.

will be produced in the cylindrical lens image. If two points lie side-by-side, a single band of double intensity results. Therefore, the band intensity at any level is proportional to the number of grains at that level, and the sum of the band intensities along a given length of the strip gives the integrated number of grains in the corresponding area of the autoradiograph. A plot of the linear distribution of band intensities along the strip gives a visual record of



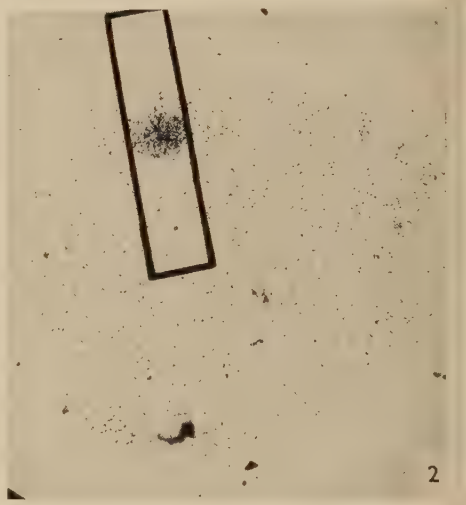


Fig. 1. Phase contrast photograph of *Amoeba proteus* labelled with adenine-C^{14}. Nucleus in upper left corner. (× 211.)
Fig. 2. Positive print of photograph of autoradiograph of amoeba shown in Fig. 1, taken from Kodalith negative used for the records shown below. Scanning path marked.

variations of grain densities that may be related to the structure of the object that produced the autoradiograph. Essentially, what we are doing is converting a two dimensional pattern of points to a one dimensional pattern of lines which may be scanned more readily. The grain distribution in one dimension is registered as numbers of lines, in the other dimension as intensity of lines.

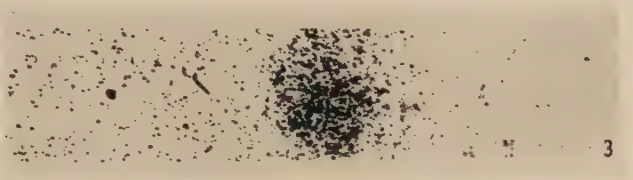
Application

The first version of the method, which we shall describe in some detail, achieves technical simplicity by the use of two photographic steps. This simplification with respect to apparatus admittedly introduces the complications of interpretation inherent in using photography for photometric purposes. The procedure as described is designed to compare the radioactivities of intracellular structures (e.g. nuclei) with each other and with their background (e.g. cytoplasm).

(1) The autoradiograph is prepared by the stripping film technique of Doniach

and Pelc [3]. Recently, Taylor and McMaster [6] have published additional details of the technique. (See also [2].)

(2) A photomicrograph is taken of the autoradiograph. We have found that if the autoradiograph is mounted in immersion oil under a cover glass, avoiding swelling of the gelatin, all of the grains in the thin emulsion may be brought into common focus with an objective of adequate focal depth. For many purposes, an ordinary 10 × objective will do. We use a 20 × apochromat equipped with an iris diaphragm.



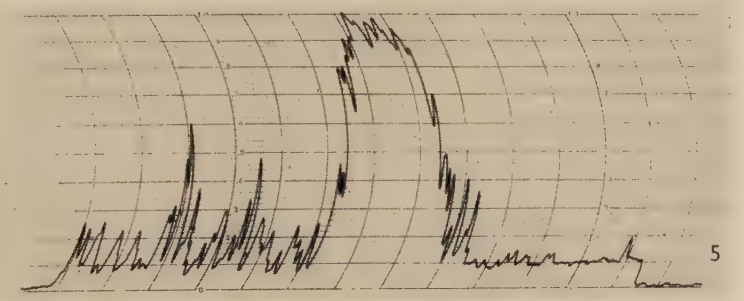


Fig. 3. Enlarged view of strip of negative scanned, taken through camera with cylindrical lens out of position.
Fig. 4. Enlarged positive view of cylindrical lens pattern of same strip as is shown in Fig. 3. This print reproduces the band pattern as it is used for microdensitometric scanning. Detail has been lost in reproduction.
Fig. 5. Microdensitometer record of pattern shown in Fig. 4. Reduced photographically to show correspondence between densitometric record, band pattern (Fig. 4) and grain pattern (Fig. 3).

If an extremely high contrast film is employed, with appropriate processing, a negative may be obtained in which the images of the grains are clear spots against a black background. The tissue itself does not influence the image. We use for this purpose Kodalith Ortho film, processed with Kodalith Fine Line Developer. Uniformity of illumination is very important, for inhomogeneities not perceptible by eye will produce large variations in the high contrast negative.

An experiment on *Amoeba proteus* will serve as an example. The cell was incubated in adenine-C^{14} (2 microcuries per ml), fixed in 45 per cent acetic acid, flattened,

covered with stripping film, and exposed for five weeks. Fig. 1 shows the details of the preparation as seen by phase contrast. Fig. 2 is a positive of the high contrast photograph of the film over the ameba, showing the distribution of grains. It is evident that the nucleus has incorporated the adenine into acid-insoluble compounds to a greater extent than has the cytoplasm.

(3) The negative, of the grain image, is mounted in the cylindrical lens camera, positioned and masked in such a way that the light passes only through the strip that is to be scanned. In this case, we wish to compare nucleus with cytoplasm, and make the strip the width of the nucleus. The strip measured is indicated in Fig. 2. Fig. 3 is an enlarged positive of the image of the strip with the cylindrical lens out of position, and Fig. 4 is a positive of the corresponding pattern with the cylindrical lens in position. Obviously, it is a simple matter to assign the band pattern at a given level to the grain pattern at the same level, and to relate this in turn to the structure of the ameba.

(4) The grain pattern and the cylindrical lens pattern are photographed. The cylindrical lens pattern will be useful for quantitative purposes only if the relation between the intensity of the bands of light and the blackening of the emulsion is known. It is necessary to use an emulsion of suitable latitude (we use Kodak Superspeed Ortho Portrait Film), controlled development, and a density wedge as a standard.

(5) The cylindrical lens pattern (Fig. 4) is scanned by means of a microdensitometer. The system, designed by one of us (G. W. E.) employs a barrier-layer cell as detector. Its output is logarithmic, with the advantage that it relates photographic density to light energy incident on the photographic surface in a linear way. A microdensitometer tracing of the record shown in Fig. 4 is given in Fig. 5. The area under a given portion of the tracing is a measure of the relative number of grains in the corresponding area of the autoradiograph. For greater accuracy, the values should be corrected for the relation between light intensity and photographic density, which is given by the "gamma" of the photographic processing or by the record of the density wedge. The use of the latter also permits us to know when we are in a region where the relation between exposure and log density is linear.

The cylindrical lens patterns obtained may be scanned by means of microdensitometers used in spectroscopic laboratories. If it is desired only to compare selected regions, any instruments that will measure light transmission at low magnifications may be used. The chief advantages of automatic recording are (1) that it provides more complete information as to the distribution of radioactivity with respect to the structural situation under study and (2) it provides greater freedom of choice with respect to the shape and size of the areas to be measured. For instance, if we are dealing with a region of low activity, it is easy to measure a longer strip.

In our work thus far, we have measured the areas under regions of the microdensitometer tracings by cutting out the records and weighing the paper.

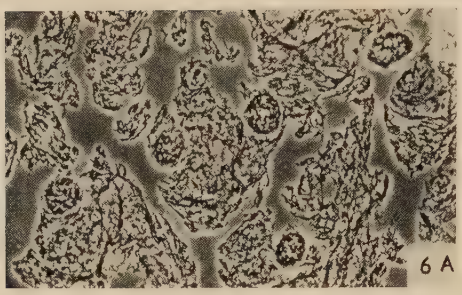
Cylindrical Lens Camera

We have adapted the Perkin-Elmer Model 38 Tiselius Electrophoresis Apparatus (Perkin-Elmer Corporation, Norwalk, Connecticut) fur use as a cylindrical lens camera merely by removing the entrance slit, the slits at the window of the ice bath, and the diagonal knife slit. The optical equivalent could be built in many forms. The negative is mounted on a vertical stage set into the chamber in which the electrophoresis cell is ordinarily placed, with a simple sliding arrangement for focussing. The light source is that built in for electrophoretic measurements.

Tests of Method

The principle of the method has been tested first by studying artificial grain patterns. These were prepared simply by placing black dots in various arrangements against a white background, and reducing the patterns by photography. The test objects were negatives in which the size of the dot images corresponded to those in the photographs of the autoradiographs. By the use of such knowns, it was established (1) that the amplitude of the record of a row of dots side-by-side was proportional to the number of dots and (2) that when the arrangements were irregular, the area under the corresponding tracing was proportional to the number of "grains". The limited response range of the photographic emulsion set limits on the range within which proportionality was observed.

A second and more realistic test was carried out by measuring the DNA (as P^{32}) of *Drosophila* salivary gland nuclei from larvae that had been grown on a medium containing P^{32} (10 microcuries per ml) as phosphate. The P^{32} was present continuously from the time the eggs were laid until the time the larvae were collecting, so that we are considering the total amount of DNA in the nucleus, in so far as this is represented by the total amount of P^{32} incorporated into DNA. Swift and Rasch (cited by Alfert [1]) had shown by the photometeric Feulgen method that the polytene salivary gland nuclei apparently doubled fairly synchronously. The measurements grouped themselves into classes. The DNA content of each class was twice that of the next lowest class. The nuclear volumes were grouped in a parallel fashion. If, then, the P^{32} accumulated in DNA in a nucleus during a given period of development measured the total DNA formed in that nucleus we would predict that the P^{32} content of nuclei of the same size class would be similar. This would serve as one test of the quantitative validity of our method.



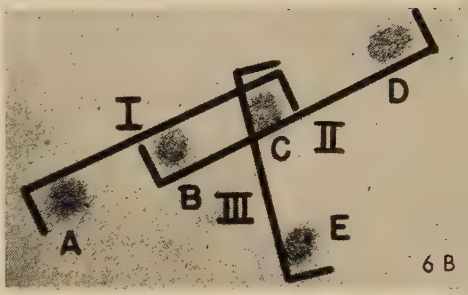


Fig. 6. *Drosophila* salivary gland preparation used for the measurements given in Table I. (A) Phase contrast photograph of region of squash containing the five nuclei which were measured. Scanning paths marked. These are relatively young nuclei. (B) High contrast negative of autoradiograph of same field. (× 81.)

The salivary glands were removed from relatively young larvae, fixed in 45 per cent acetic acid, and extracted with 1*N* HCl at 60° for 10 minutes, to remove RNAP. After the autoradiograph was prepared, a group of five nuclei of the same size class was chosen for comparison. Fig. 6 (A) is a phase contrast view of the region containing these five nuclei. Fig. 6 (B) shows the autoradiograph of the same region. In order to measure these five nuclei, three scanning paths had to be used, and these are marked on Fig. 6 (B). Nucleus B had to be scanned twice, and nucleus C had to be scanned three times, which served as a further check on the method.

The results are given in Table I. The agreement is rather good (well within 10 per cent) whether we compare the five nuclei or compare the different

Table I

Relative P^{32} activities of nuclei shown in Fig. 6. The designation of the individual nuclei and of the strips that were scanned is the same as in Fig. 6 (B).

Nucleus	Strip	Total P^{32} activity in nucleus (relative units)
A	I	65
B	I	63
B	II	67
C	I	68
C	II	72
C	III	73
D	II	65
E	III	67

measurements on the same nucleus. In fact the results show far less variation than the photometric values of Swift and Rasch, suggesting even more strongly that the duplication of these polytene chromosomes goes synchronously in a group of nuclei of the same age and must be a rather abrupt event.

The examples thus far have dealt with rather clear cut autoradiographs, where differences visible by eye were quantitated. We are certain from work as yet unpublished that the method will permit us to establish differences imperceptible to the eye, but are not yet certain as to the smallest measured difference that can be considered significant.

Advantages, Limitations and Improvements

The usefulness of any method of evaluating autoradiographs quantitatively is obvious, and we need here only compare the present approach with others.

(1) Direct visual counting of grains is exceedingly laborious, and is practicable only where the counts are low and where small areas may be dealt with.

(2) Direct microdensitometry of the autograph itself is useful, as pointed out by Levi, only when dealing with relatively large areas and fairly dense autoradiographs.

(3) There is no evidence as yet that electronic counting by the flying spot method can be accomplished simply. It seems to require observation at very high magnification, which in turn creates a problem in bringing the grains into focus. The present method requires only lower magnifications and permits all the grains to be in focus. Where observation at high magnification (oil immersion) is required by the size of the biological object, our method too will be limited by problems of focal depth.

(4) The nuclear track method, recently discussed in detail by Levi, is limited, for reasons that she sets forth fully, to isotopes emitting particles of low energy and to free cells that are small with respect to track lengths. These limitations do not apply to "grain pattern" autoradiographs on thin emulsion.

(5) A method that can scan large areas and still report local detail has potential advantages in practice over one that involves sampling of small areas one at a time, such as the flying spot system, or visual counting.

The most obvious sources of error in the version of the method that has been described lie in the photographic steps. The means of eliminating the second photographic step, on which the photometry depends, are apparent,

and are being developed by us now. It is only necessary to place the negative image of the grain pattern on a moving stage, and to scan the travelling strip by means of a photoelectric recording system placed behind the camera. We are also developing a system that will eliminate the first photographic step by scanning directly a dark field image of the autoradiograph itself.[1]

Ultimately, it will be necessary to apply any grain counting method to absolute measurements of radioactivity. Whatever method of counting is used, the immediate solution seems to be calibration by standards of known activity.

It is conceivable that the cylindrical lens could be eliminated in favor of direct photoelectric registration of the light signals from the individual grains. However, the use of the cylindrical lens seems to be advantageous practically, because it compresses the information into one dimension.

SUMMARY

An optical system for scanning autoradiographs is described. It is based essentially on forming a band pattern of the grains in a strip of the autoradiograph by means of a cylindrical lens camera and on the photoelectric recording of the distribution of band intensities along the strip.

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[1] *Footnote added in proof.* Both of the improvements mentioned have now been accomplished, the former by the present authors, the latter by J. E. Gullberg. They will be published shortly. The present method retains the asset of simplicity of instrumentation.

Experimental Cell Research, **9**, *313–318 (1955)*

STUDIES IN THE MATURATION OF UNDERRIPE SEA URCHIN EGGS

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It has been observed that sea urchin eggs often are very resistant to artificial insemination. Eggs with the polar bodies still remaining in the jelly are especially difficult to fertilize. As a rule, the jellies of these eggs are very compact which constitutes a certain obstacle to sperm-entrance. Upon insemination underripe sea urchin eggs are characterized by giving a low percentage of fertilization, low irregularly elevated membranes, strong contraction of the egg surface and incomplete cleavage. The ensuing development is abnormal and the resulting plutei have short arms. There seems also to be a certain tendency for polyspermy in underripe eggs [cf. 7, 8]. The refractoriness of the egg with respect to fertilization has been ascribed to a cytoplasmic underripeness [cf. 6]. Many investigations have been carried out in order to find methods for removing this state of underripeness. Runnström *et al.* [7, 9, 10] have shown that the fertilization of underripe eggs is improved upon the addition of serum albumin, amino acids and sodium periodate. However, it has been pointed out that the substances improving fertilization have a beneficial effect on the sperm [11, 13]. In all the experiments referred to above fertilization was carried out in the presence of the improving substance, thus the fertilizing capacity of the sperm was also improved. Therefore, Tyler has claimed that there is no underripeness of the egg and he ascribes the improvement in fertilization obtained after treatment with amino acids and chelating agents to an improvement of the sperm [12].

However, it was shown that pretreatment of underripe eggs with albumin, glycine and sodium periodate increases the fertilization rate and the total percentage of fertilized eggs [3]. Furthermore, it was demonstrated that the presence of the improving substances at insemination enhanced fertilization still more. These experiments clearly indicate the relative role of improvement of the sperm and of the eggs.

Runnström described an experiment (cf. [6], Fig. 1) in which underripe eggs from *Psammechinus miliaris* were stored in normal sea water without

any additions. The result is very striking. Eggs inseminated immediately after their removal from the ovaries exhibited the characteristics of underripeness mentioned above. Part of the unfertilized eggs were stored in sea water and aliquots were inseminated after about two, six and ten hours. After two hours the signs of underripeness were less evident. After six hours fertilization was optimal with well elevated smooth membranes. At the last insemination (after ten hours) the eggs had already begun to die and only a minor part became fertilized. This experiment is a proof of underripeness, the existence of which has been denied [12]. This paper presents some new results obtained by aging the eggs in sea water.

MATERIAL AND METHODS

This work was carried out at the Zoological Station of Kristineberg on the Swedish west coast, during July–August 1954. In all the experiments *Psammechinus miliaris* served as material. Both the littoral form (Z-form) and the deep form (S-form) were used [cf. 5]. The experiments were performed according to the fertilization rate method described previously [3] in which the sperm is eliminated by means of sodium lauryl sulphate. The "dry sperm" was kept in a refrigerator at $+4°C$[1] and a new sperm suspension was prepared for every insemination. The concentration of sperm was 1.5×10^{-6}/ml in all the experiments.

EXPERIMENTAL

Sea urchins were opened and the ovaries put into a beaker containing sea water. The eggs were removed from the ovaries by passing the contents of the beaker through fine silk cloth. The eggs were washed once by sedimentation and the first fertilization rate experiment was performed within the course of 15 minutes after the animals were opened. The major part of the egg suspension was stored in the laboratory at a temperature of about 17°C. The eggs were allowed to stand in the original sea water without further washing with the exception of the experiments with periodate. In these latter experiments the eggs were pretreated with periodate and washed once with sea water before insemination; the corresponding untreated control was washed with the same volume of sea water.

A new fertilization rate experiment was made about every hour until a significant decrease in fertilization rate was observed. In some experiments part of the eggs were pretreated with sodium periodate. The concentration of periodate was $2 \times 10^{-5}M$–$2 \times 10^{-6}M$. In other experiments jelly-free eggs

[1] The influence of temperature on the sperm has also been studied and will be described in a forthcoming paper.

were used. The eggs were freed from the jelly coat by treating the eggs for 3 minutes with HCl at a pH of 5.6–5.8. About 60 experiments were made.

The results reveal that the eggs seldom show optimal fertilizability upon removal from the ovaries. There is a marked increase in fertilization rate up to an optimum of 3–5 hours after the eggs were shed followed by a rapid decline. Figs. 1 and 2 show two typical experiments. Eggs with intact

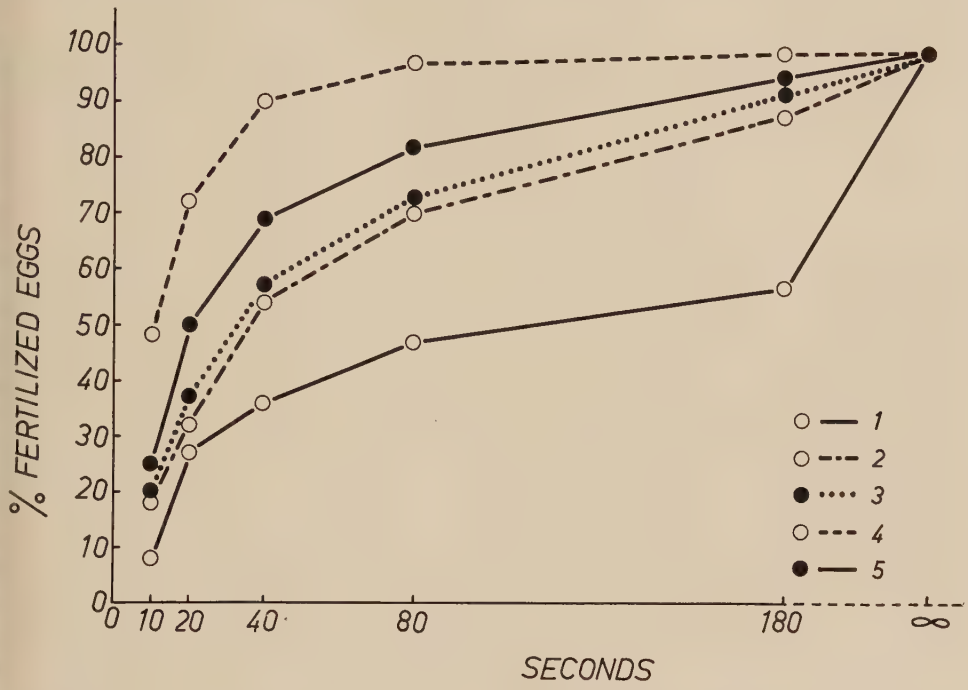

Fig. 1. Eggs from *Psammechinus miliaris*. Eggs with jelly coats.

(1) Control. (2) The eggs had been stored in sea water for 1 hour before insemination. (3) The eggs had been stored in sea water for 2 hours before insemination. (4) The eggs had been stored in sea water for 3 hours before insemination. (5) The eggs has been stored in sea water for 4 hours before insemination. 10, 20, 40 ... 180 seconds after insemination sodium lauryl sulphate was added to a final concentration of 0.001 per cent.

jelly coats and jelly-free eggs give the same improvement when aging in sea water. The experiments with periodate showed that it is possible to get an improvement of the eggs beyond that obtained simply by aging the eggs in sea water. Furthermore, it was found that eggs which had already passed their "sea water optimum" and had begun to decline could be raised to a higher fertilization rate (cf. Fig. 2).

The observations on membrane elevation, cleavage and ensuing development showed that these characteristics also were improved upon storing the eggs in sea water. There was a close correlation between fertilization rate and segmentation. Thus the eggs fertilized directly after the removal from the ovaries often showed nuclear division without ensuing cytoplasmic cleavage. In eggs stored for about 2–5 hours the cleavage was quite normal.

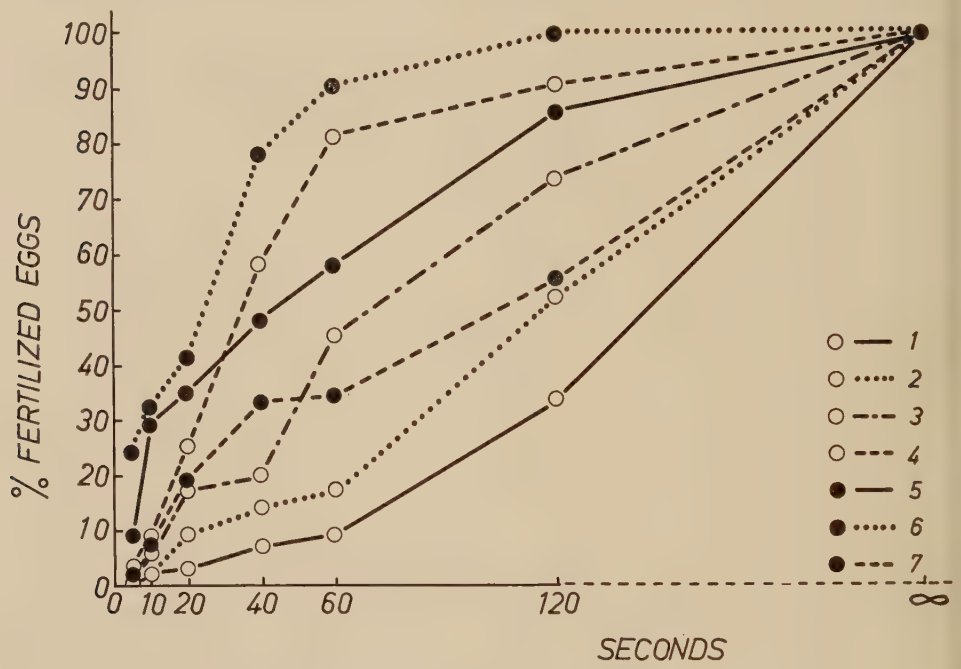

Fig. 2. Eggs from *Psammechinus miliaris*. Eggs with jelly coats.

(1) Control. (2) Before insemination the eggs were pretreated for 10 minutes with periodate ($2 \times 10^{-5}M$). (3) The eggs had been stored in sea water for 1.5 hours before insemination. (4) The eggs had been stored in sea water for 2.5 hours before insemination. (5) The eggs had been stored in sea water for 4.5 hours before insemination. (6) The eggs had been stored in sea water for 4.5 hours. Before insemination the eggs were pretreated for 10 minutes with periodate ($2 \times 10^{-5}M$). (7) The eggs had been stored in sea water for 5.5 hours before insemination. 5, 10, 20 ... 120 seconds after insemination sodium lauryl sulphate was added to a final concentration of 0.001 per cent.

DISCUSSION

The present results show that underripe eggs become ripe when stored in sea water. Tyler [12] claimed that the improvement obtained by treating the egg with albumin, glycine, periodate etc. could not be interpreted as due to

a ripening of the egg. Furthermore, he stated that pretreatment of the egg did not significantly improve the fertilization. As was previously pointed out Tyler's own results do not seem to be in agreement with his conclusions. They indicate that pretreatment of the egg improves fertilization [3]. Tyler's interpretation of the effects obtained by treating the sperm with glycine, versene etc. ([12] Tables III, VII) seems to be open to certain objections. As far as it is possible to judge from his data eggs were fertilized with sperm which had been treated with sea water containing the agent to be tested. There is no statement made as to whether or not the treated sperm were washed with sea water before insemination. Therefore, it may be assumed that fertilization occurred in the presence of e.g. glycine. Tyler measured the total percentage of fertilized eggs and the percentage of good and poor membranes, but did not measure the rate of fertilization and there is no information about when the fertilizing sperm enters the egg. Thus Tyler's data do not exclude that the eggs were improved by an action of the added agents before fertilization took place. The concentrations of e.g. glycine [12] were quite sufficient to improve fertilization even after the dilution which occurred at insemination [cf. 3].

In his excellent study of the amino acid metabolism in developing sea urchin embryos Kavanau [4] showed that considerable changes take place in the amino acid fractions of unfertilized eggs when the eggs are aged in sea water. Kavanau assumed these changes to be correlated with the ripening of the egg. (For discussion cf. [4] pp. 545–549). He also pointed out that there is a close correlation between his own results and Borei's determinations of the oxygen consumption of unripe and ripe eggs [1, 2]. These studies [1, 4] give us a certain insight into the biochemical background of the phenomenon referred to as cytoplasmic maturation. On the other hand, this phenomenon may be followed as shown above rather exactly by the method of determining the rate of fertilization.

SUMMARY

When unripe sea urchin eggs are aged in sea water they become mature. Membrane formation, cleavage and development are improved. The fertilization rate is low in unripe eggs but is increased upon storage in sea water.

I wish to express my sincere thanks to Professor J. Runnström for suggesting this problem to me and for his interest in the work. I am also indebted to Dr. G. Gustafson, Kristineberg, for working facilities and generous supply of material.

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ZENTRIFUGEN- UND POLARISATIONSMIKROSKOPISCHE UNTERSUCHUNGEN AM NUCLEOLUS *IN VITRO*

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Durch neuere Fortschritte der topochemischen Zellforschung stark in den Vordergrund wissenschaftlicher Analysen gerückt ist der Nucleolus. Zu seiner topochemischen und leptonischen Analyse an intakten Zellen und zur Verfolgung seiner Lebensäusserungen und ihrer Kontrolle an gefärbten Präparaten bieten Gewebekulturen *in vitro* besonders günstige Bedingungen [29]. So wurde, wie zusammenfassende Übersichten [14, 16, 22, 23, 39, 41, 43] erkennen lassen, der Nucleolus neuerdings nach vielen Gesichtspunkten studiert, so etwa hinsichtlich amöboider Beweglichkeit (C. C. Macklin), des Zusammenfliessens und Zerteilens (T. S. P. Strangeways, R. C. Page, W. H. Lewis, H. B. Fell, A. F. Hughes), der Veränderungen von Grösse, Form, Zahl, Lage und Anfärbbarkeit (J. J. Biesele, R. Thorspecken, R. Lettré), der Beeinflussung durch Hyper- und Hypotonie, Acidität, Vorkommen von Antikörpern (L. Stockinger), verwickelter enzymatisch regulierter Prozesse des Stoffaustausches (W. H. Lewis, R. J. Ludford, J. Saguchi, R. Lettré) usw. Bisher kaum in Angriff genommen ist aber meines Wissens die *indirekte leptonische Strukturanalyse des Nucleolus mittels polarisierten Lichtes.* Auf solche Weise hat nur Monné [24] für Oocyten und Leydigsche Blasenzellen von *Helix*-Arten das Vorkommen einer negativ doppelbrechenden Membran des Nucleolus beschrieben. Vor allem aber planmässige quantitative Bestimmungen von Doppelbrechung, accidentellem Färbungsdichroismus und Beugungspolarisation fehlen bislang. Mit dem Stande der so fundierten Leptonik des Nucleolus mag sich daher die folgende Mitteilung befassen. Wegen einer gewissen Unbestimmtheit der herrschenden Nomenclatur sei noch erwähnt, dass hier unter dem Nucleolus wie bei Lettré und Siebs [21] die Gesamtheit der mit Methylgrün anfärbbaren Strukturen chromosomaler Natur und der mit Pyronin darstellbaren amorphen Substanzanteile verstanden wird.

MATERIAL UND METHODIK

Material.—Nach einem Verfahren von Haberlandt [15] ist 36–40^{h} nach Injizieren von 0,3 ml einer mässig dichten Suspension von sterilem Kohlepulver in Ringer in

den Lymphsack von *Rana fusca* die noch klare, an Lymphocyten angereicherte Lymphe entnommen und eine 10 % Lsg. von Gelatine puriss. in Ringer (Zusatz von etwas Blutserum des Tieres) hinzugefügt, der sich bildende „Gewebeschleier" mit steriler Pinzette abgehoben, mit Ringer gespült, zerschnitten und explantiert worden. Nach 24–30^h hat sich um das Explantat ein „Hof" migrierender Zellen gebildet, und nach einigen Passagen resultieren endlich „Reinkulturen" einkerniger Leukocyten. In einem Plasmakoagulum sind nach A. Maximows Vorschriften double-cover slip and hanging-drop cultures ([26], S. 147) unter Verwendung von L. E. Blakers Medium für Monocyten ([26], S. 119) angesetzt worden.

Bei der *phasenmikroskopischen* Untersuchung wird für ungefärbte Leukocyten das mittlere Dunkel-A$^-$ Beugungsplättchen, für gefärbte das 1 B–0.25 λ Plättchen andern optischen Zusammenstellungen vorgezogen [1]. Der dabei nicht zu vermeidende helle Lichthof um die Zellkonturen bleibt aus (Abb. 1) bei Anwendung einer *anoptralmikroskopischen* Anordnung nach Wilska [44] oder einer dem *Dyson Interferometermikroskop* nachgebildeten Versuchsanordnung [34]. Gelegentlich sind auch Prinzipien der Phasenmikroskopie und der *Polarisationsoptik* nach Arbeitsvorschriften von Osterberg [25] kombiniert worden, um so die Phasendifferenz von $-\pi$ bis $+\pi$ und das Amplitudenverhältnis von 0 bis ∞ zu variieren [30]. Teilweise ist ferner mit einer Anordnung als Zentrifugenmikroskop [27] nach der Bauart von E. Newton Harvey und A. L. Loomis bei konstanten Beschleunigungen zwischen 4200 und 4500 g die Schichtung in den Leukocyten und ihr Einfluss auf den Nucleolus phasen- und polarisationsmikroskopisch verfolgt worden.

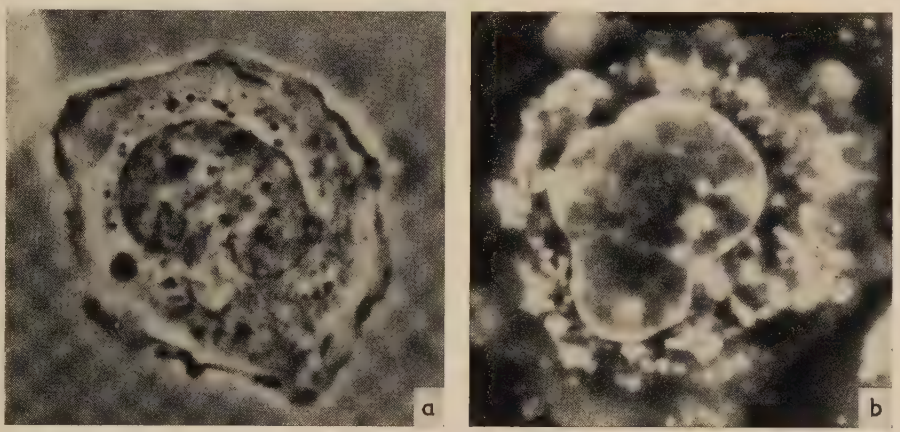
Teils sind ungefärbte Leukocyten benutzt, teils ist nach der von Chayen [8] entwickelten Methode mit Methylgrün-Pyronin oder nach Kurnick [20] allein mit Methylgrün *gefärbt* worden. Zum Vergleich sind Nucleolus und Mitochondrien nach Semmens and Bhaduri [36] nach Färben mittels Methylgrün gegen die Feulgenreaktion differenziert oder nach Joyet-Lavergne [19] elektiv mit Leukoderivatindigosol dargestellt worden. Bei Durchführen der meist angewandten Methylgrün-Pyroninfärbung kann nach K. Helly mittels Zenker mit Formol statt Eisessig fixiert werden.

Die Messung der *Doppelbrechung* (Δn) ist gewöhnlich wie bisher [32] nach Braces Verfahren mit einem Kompensator variablen Azimuts und konstanter Retardation (λ/30-Glimmerplatte) und einem drehbaren Halbschattenkeil nach Macé de Lepinay ([28], S. 55, 64) vorgenommen, in vereinzelten Fällen auch aus Retardationskurven nach der densitometrischen Anordnung von Swann and Mitchison [40] erhalten worden. Zur quantitativen Bestimmung des *Dichroismus* (Δk) nach ausgeführter Methylgrünfärbung sind zur Errechnung der Differenz k_a-k_0 die beiden Absorptionskoeffizienten k_a, k_0 nach einer Methode Bereks wie bei den vorangegangenen Versuchen [31] mit seinem Mikrophotometer einzeln ermittelt worden. Maximale Δk-Werte erhält man bei Methylgrünfärbung im roten Spektralbereich (600–720 mμ), bei Methylenblaufärbung im Gelb (550–590 mμ) durch Gebrauch der betreffenden Lifa-Filter.

BEFUNDE AN DEN LEUKOCYTEN

Phasen- und anoptralmikroskopisch weist das Plasma der *Leukocyten in vivo* (Abb. 1) bleiche, stark lichtbrechende, chromophobe Granula auf, welche sich manchmal in lebhafter Brownscher Bewegung befinden und

undulierende Randbewegungen oder zuweilen die Ausbildung amöboider Protrusionen zeigen. Der meist ziemlich chromatinreiche Kern ist nicht immer kugelig, sondern kann auch stab-, lappen- oder hufeisenförmige Gestalt annehmen oder bisweilen septiert sein. Nur sehr selten sind wie an Leukocyten von *Triton* [9] Mitosen beobachtet worden.



Abbl. 1. Ein Leukocyt mit Kern und Nucleolus in a) phasenmikroskopischer, b) anoptralmikroskopischer Aufnahme.

Der im allgemeinen scharf konturierte *Nucleolus* ist *in vivo* stark lichtbrechend, farblos oder trübe und zufolge seiner Begrenzung und seiner Formänderungen flüssig bis viscos. In manchen Fällen zeigt er an der Oberfläche eine dünne Schicht verstärkter Lichtabsorption. Es kann aber nicht entschieden werden, ob damit das Vorkommen zweier chemisch differenzierter, konzentrisch verteilter Phasen [7, 11] bestätigt wird. Gegenüber andern phasenmikroskopischen Untersuchungen *in vitro* kultivierter Leukocyten, wie etwa solchen der menschlichen Nasenmucosa [35], ergeben sich also keine besonderen Eigentümlichkeiten.

Die *zentrifugenmikroskopisch* gefundene Schichtung der Leukocyten (Abb. 2) lässt neben Lagen von allerhand Granula, Mitochondrien, Dictyosomen (Mikrosomen) und Lipoidtropfen im Hyaloplasma eine Verlagerung und Deformation sowohl des Kernes als auch des Nucleolus erkennen. Da über die noch im Gange befindlichen *cytochemischen* Untersuchungen später berichtet werden soll, genügt hier der Hinweis, dass in Bestätigung von Vincent [42] das Auftreten von Histonen, Phosphorproteinen [6], Ribonucleoproteiden [2, 5] und alkalischer Phosphatase [10] wahrscheinlich zu machen ist, das Vorkommen von Lipoiden [13, 37] und Polysacchariden [38] aber einer späteren Entscheidung überlassen bleiben muss.

Auch mit empfindlichen Nachweismethoden zeigt der Körper der *Leukocyten in vivo* keine Δn. Die optische Isotropie verschwindet oft auch dann nicht, wenn die Leukocyten der Kulturen zum Agglutinieren gebracht werden. Aber nach Einbetten in Glycerin (n_D 1,456) oder in Apáthys Gummi arabicum-Zucker-Sirup und besonders nach starkem Austrocknen der agglutinierten Zellen ist die Δn nicht mehr zu übersehen und schwankt je

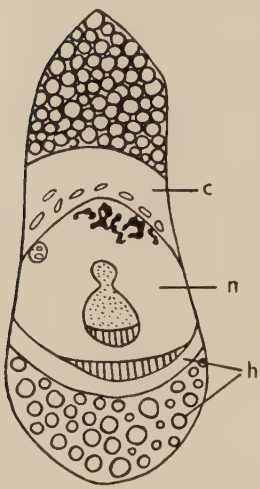


Abb. 2. Zentrifugenmikroskopische Schichtung der Leukocyten: *c*, Cytoplasma mit Mitochondrien, *n* Nucleus mit Chromatinbrocken (Chromosomen?) und zentrifugal aufgegliedertem Nucleolus, *h* Hyaloplasma.

nach den besonderen Versuchsverhältnissen zwischen 4,4 und 6,8 mμ. Das die Anisotropie bedingende Material ist anscheinend in oberflächlichen Zellschichten lokalisiert und kann meist mittels lipophiler Flüssigkeiten extrahiert werden. Austrocknungsversuche mit $CaCl_2$-Lösungen oder Phosphorsäureanhydrid rufen keine accidentelle Anisotropie der Leukocyten, wegen zu sehr forcierter Wirkung also wohl keine genügende Orientierung anisodimensionaler Leptonen hervor. Durch Einwirken von Chloroform, Äthyläther oder Gemengen beider mit Essigsäure wird die Anisotropie der agglutinierten Leukocyten verringert und endlich ganz aufgehoben. In den zentrifugenmikroskopischen Versuchen ergibt sich mit zunehmender Dauer und Beschleunigung der Schleuderung die nach früheren Erfahrungen erwartete Zunahme der Anisotropie der Randschichten und des deformierten Nucleolus. (Abb. 3).

In polarisationsoptischer Hinsicht im ganzen den Leukocyten ähnlich verhalten sich vergleichsweise untersuchte Amöbocyten von Schnecken (vgl. auch [12]).

UNTERSUCHUNGEN AM NUCLEOLUS *IN VITRO*

Der Nucleolus zeigt Δn nur an seinem Rande, nicht auf der Fläche. Seine Membran lässt *polarisationsoptisch* zwischen gekreuzten Nicols ein negatives Kreuz erkennen, d.h. die Länge der Indexellipsenachse verläuft tangential. Die Intensität der Δn is weitgehend abhängig von der Dicke der

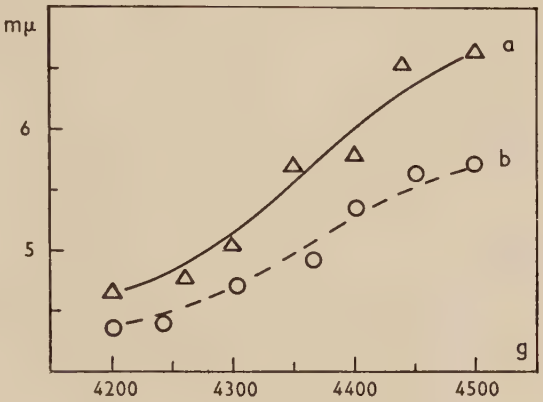


Abb. 3. Zunahme der Anisotropie (mμ) proportional der Zentrifugalkraft (g) bei a) der Randschicht desikkierter Leukocyten (△——△), b) einem Nucleolus (○----○).

Begrenzungsschicht. Durch Einwirkung von Formol wird die Stärke der Δn nicht verändert, durch die von Äthylalkohol manchmal ein wenig erhöht. Durch Imbibieren der Leukocyten-Kulturen mit Medien von einem Brechungsindex höher als Ringer, wie Zucker- und Harnstofflösungen, erfolgt eine je nach dem Refraktionsverhalten des Imbibitionsmediums wechselnde Verringerung der Δn. Beim Imbibieren mit Canadabalsam verkehrt sich das negative in ein positives Kreuz. Zentrifugenmikroskopisch lässt sich auch am Nucleolus die Zunahme der Δn mit der Schleuderungsrate zeigen (Abb. 3).

Nach diesen Versuchen ist die *Δn des Nucleolus negativ einachsig mit radialer optischer Achse.* Welche Folgerungen über seine Leptonik aus diesem Befund zu ziehen sind, wird die Diskussion zeigen.

Wie doppelbrechende Objekte oft so zeigt auch der methylgrün-gefärbte Nucleolus der Leukocyten ausserdem *Färbungs-Δk.* Die so auf andere Weise mögliche Bestimmung der Indexellipse im ungefärbten Nucleolus ergibt die Bestätigung ihrer tangentialen Orientierung. Bei vorsichtigem Ansäuern geht der Färbungs-Δk von dem normalen Farbwechsel grünlich-blau → blau-violett allmählich in die Farbfolge grünlich → rot-violett über.

Durch die Reaktionsverschiebung bleibt also der Δk erhalten. Nach angestellten Messungen ändert sich auch im Ansäuerungsversuch trotz Beeinflussung der Stärke der Δn die des Δk nicht nennenswert. Auffällig ist, dass in einer Reihe von Präparaten der Nucleolus stellenweise trotz starker Farbstoffaufnahme keinen nennenswerten Δk erkennen lässt, doch lässt sich durch genaue Vermessung dieser Stellen zeigen, dass sie auch keine Δn aufweisen.

DISKUSSION DER ERGEBNISSE

Es möchte sich empfehlen, bei diesen wie bei andern Analysen der Leptonik biologischer Objekte die *terminologische* Gliederung Bretschneiders [3] anzuwenden (Abb. 4). Danach erfassen die polarisationsoptisch fundierten Untersuchungen zum Unterschied von elektronenmikroskopischen Befunden noch nicht die Molekeln sondern erst micellare Grössenordnungen, wie *Leptonen* im Sinne von Pfeiffer [27] und von Bretschneider [3], während in den Leukocyten gefundene Mikrosomen und „Granula" Bretschneiders „Synergon", die Mitochondrien zusammen mit den hier nicht beobachteten Chromosomen, Sarcomeren und ähnlichen cytoplasmatischen Formgebilden und vielleicht auch schon der Nucleolus als ganzes seinen „*Cytomeren*" zuzuordnen sein würden.

Indem das Vorkommen von Lipoiden nicht allein cytochemisch, sondern auch aus dem Nachweis von Myelinfiguren nach Extraktionsversuchen wahrscheinlich gemacht werden kann, dürfte sich bei den *Leukocyten* nach dem Charakter der Δn (positiv zur Flächennormale) die beim Agglutinieren und Austrocknen der Zellen auftretende *accidentelle* Δn durch senkrecht zur Zelloberfläche orientierte Lipoidmolekeln ergeben. Die übereinstimmende Wirkung von Glycerin und Apáthys Sirup würde sich dann aus Wasserentzug und dabei resultierender Verbesserung der Orientierung des Proteinsubstrats erklären, das für die geordnete Einlagerung freiwerdender Lipoide anzunehmen ist. In solchem Sinne würde sich dann das Auftreten accidenteller Anisotropie der Leukocyten andern bekannten Phänomenen, wie *Lipo-* oder *Mucophanerose*, vielleicht auch gewissen pathologischen Vorgängen bei *Amyloidosen* [18], in den Grundzügen ähneln.

Aus der Wirkung lipophiler Mittel auf die Intensität der Δn *des Nucleolus* ist auf ein auch cytochemisch gesichertes Vorkommen von Proteinen in der Aussenschicht zu schliessen. Dann folgt aus dem negativen Charakter der Δn mit Wahrscheinlichkeit, dass die Aussenmembran der Gebilde aus einer

Folie von Proteinen aufgebaut wird, in welcher Leptonen oder Fadenmolekeln der Polypeptide parallel zur Oberfläche, aber desorientiert gegen benachbarte Leptonen gelagert sind, d.h. dass die Aussenschicht des Nucleolus leptonisch einer unter Binnendruck stehenden Kugelschale gleicht und an in allen tangentialen Richtungen konstante Spannungen adaptiert ist. Der Einfluss stärker lichtbrechender lipophiler Mittel weist auf den *Einbau von*

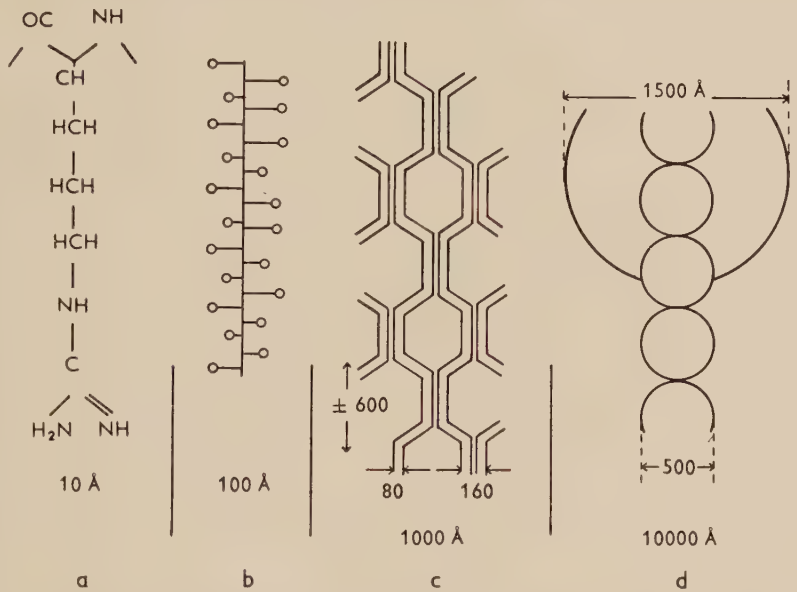


Abb. 4. Diagramm in logarithmischer Grössenordnung aufeinanderfolgender Strukturen zur Veranschaulichung der Nomenklatur Bretschneiders: a) höher polymerisierte Proteinkette (Peptidbindung), b) Proteinmolekel, c) Lepton, d) Interchromidialkette und Chromidium.

Lipoiden in die Folie hin, und nach allen bisherigen Erfahrungen können diese nur senkrecht zu den Proteinmolekeln, also ihrer Länge nach radial, eingeordnet sein. Polarisationsoptisch begründete Anzeichen für das Vorkommen quergestellter Lamellen im Nucleolus, wie sie bei Mitochondrien nachgewiesen worden sind [33], haben die Versuche nicht ergeben. In welcher Weise ein so geordnetes, peripher gelegenes Protein-Lipoidsystem den Stoffaustausch von Kern und Nucleolus und vielleicht auch eine Verlagerung nucleolarer Substanz gegen das Cytoplasma [17, 21] zu lenken vermag, gilt es an dieser Stelle noch nicht zu untersuchen (vgl. [4]). Die Umkehrung des optisch negativen Kreuzes in ein positives beim Imbibieren mit lipophoben Medien beweist entsprechend G. F. Göthlins ,,metatroper

Reaktion" die vorherige Überkompensierung der Δn (entgegengesetzten Charakters) des Lipoidanteils über die Δn des Proteins. Die Reduktion der Δn nach Einwirken stark lichtbrechender, gegen Lipoide indifferenter Medien deutet auf ein Zusammenwirken von *Eigen- und Form-*Δn hin, ohne dass aber wegen Unkenntnis des genauen Kurvenverlaufes in geeignet abzuwandelnden Imbibitionsversuchen der Anteil beider quantitativ gegeneinander abgegrenzt werden könnte.

Wenn trotz ausreichender Färbungsintensität stellenweise der Δk ebenso wie die Δn ausbleiben, so fehlt den Farbstoffteilchen an jenen Stellen die Ausrichtung, vermutlich infolge ungenügender Orientierung des Substrats (*Destruktion des* an andern Stellen wohlausgebildeten *Foliensystems*?). Daraus könnte geschlossen werden, dass der *Färbungs-*Δk allein auf Eigen-Δk der Farbstoffteile beruht. Gewisse Veränderungen bei Zutreten quellend wirkender Medien zeigen jedoch, dass *Form-*Δk durch Methylgrün in den Versuchen nicht sicher ausgeschlossen worden ist; in solchen Fällen müsste angenommen werden, dass der Farbstoff an dichroitisch gefärbten Stellen des Nucleolus wegen der Anisotropie und regelmässigen Orientierung der interleptonischen Räume geordnet adsorbiert wird, an den andern aber nur in fest-gelöster Modifikation vorliegt. Eine Klärung dieser *Unsicherheit* (vgl. auch [32]) kann aber erst bei Fortsetzung der Versuche zu erbringen erstrebt werden.

SUMMARY

(1). Leucocytes cultivated *in vitro* in double cover slip and hanging-drop cultures have been investigated by different recent methods, including centrifuge microscopy and polarization optics. In regard of the terminology of the organelles of living cells the nomenclature applied by Bretschneider [3] is commended.

(2). The *leucocytes* mostly show isotropy unless they are subjected to certain experiments of desiccation which make possible an orientation of the leptones. The accidental anisotropy of desiccated leucocytes is compared with phenomena like lipophanerosis, mucophanerosis and some pathological processes succeeding amyloidoses.

(3). The *nucleolus* of the leucocytes shows monaxial birefringence with radial optical axis. If the leucocytes are stained with methyl green the stain-dichroism substantiates the tangential orientation of the index ellipse.

(4). From the optical behaviour of the nucleolus it is to be concluded that its surface layers are built up by a foil consisting of protein leptones parallel with the surface and of enclosed lipid molecules, that is to say, the surface layer of the nucleolus appears as a spherical skin with constant expansions in all tangential directions.

(5). The anisotropy of the nucleolus results by co-operation of intrinsic and form birefringence, but a quantitative delimitation between them is still unknown. Whether after staining form dichroism does occur beyond intrinsic dichroism is also to be decided in the future.

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RELATIONS BETWEEN CELL GROWTH AND CELL DIVISION

I. REDUCED WEIGHT, CELL VOLUME, PROTEIN CONTENT, AND NUCLEAR VOLUME OF *AMOEBA PROTEUS* FROM DIVISION TO DIVISION[1]

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The lack of precise information concerning the growth and progressive physiological and chemical differentiation of the cell over its life cycle is attributable largely to difficulties involved in accurate single-cell measurements and to the inavailability, until very recently, of techniques for synchronizing the growth and division of cells in a homogeneous population. The new methods for obtaining populations of synchronously growing and dividing *Tetrahymena* [15], bacteria [2, 5], and *Amoeba proteus* [3] will permit the introduction of standard techniques of measurement in the study of the cell life cycle.

Unfortunately, most of the older studies on volume growth of individual protozoa suffer either from incompleteness, lack of control over culture conditions, or inaccurate methods of measurement and are consequently of limited interest. Popoff's careful determination [11] of the cell and nuclear growth curves for the ciliate, *Frontonia leucas*, constitutes an exception. Growth in volume of this organism is most rapid directly after division, falls to a lower rate during most of the life cycle, and rises slightly just before division. The slight increase before division is a reflection of the rapid increase in macronuclear volume during the period just preceding cell division. Volume changes, however, do not necessarily reflect similar changes in the metabolic machinery and should be checked against other types of measurement.

Zeuthen [19] has been able to follow the respiration of *Tetrahymena* starting with one cell and continuing measurements as the number of cells increased exponentially during the subsequent synchronous life cycles.

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Zeuthen has used the rate of respiration as a measure of the amount of respiring material and therefore as an indication of growth and finds that growth is linear through most of the cycle but ceases completely a short time before division.

In the experiments described below, growth of *Amoeba proteus* has been followed over the cell cycle using several types of measurement. Some of the studies were made on single cells but for the most part groups of synchronously growing cells were employed. Shortly after these experiments were completed, an interesting paper by Swann [16] appeared in which the need for information concerning cell cycle growth was stressed. In his discussion of cell growth and division Swann presents a hypothetical growth curve for the cell which finds striking experimental confirmation in the studies to be reported here.

METHODS

Amoeba culture.—In the rice or wheat type of amoeba culture, amoebae grow and divide at variable and unpredictable rates, their fates depending upon the growth of all the other organisms present. Until a pure culture method is developed, the best compromise is controlled feeding with organisms of known history grown separately. Therefore, the usual culture method was replaced by a system in which the amoebae are fed exclusively on *Tetrahymena.* The *Tetrahymena* are grown separately and are added to the amoeba cultures as required. There is no other source of nutrient for the amoebae. Control of cultures and standardization of conditions are much improved, and the amoebae form a physiologically uniform population. For details of the culture method see [13].

The inorganic amoeba medium consisted of the following: KCl, 6 mg; $CaHPO_4$, 4 mg; $MgSO_4$, 2 mg; 1000 ml pyrex distilled water.

Growth measurements. –Growth of amoebae over the complete life cycle was measured in terms of reduced weight, protein, cell volume, and nuclear volume. It was possible to follow the growth in reduced weight of single amoebae. Growth in protein and volume were followed by making measurements on groups of synchronously growing amoebae fixed at known points of the cell cycle. From these data growth curves were constructed. The groups of synchronously growing amoebae were obtained by collecting several hundred dividing amoebae from stock cultures and culturing the resulting post-telophase cells together on *Tetrahymena* in the dark at 23°C on an agar surface. Under these conditions the amoebae divide after 24 hours. The synchrony is satisfactory but not perfect. All the cells divide over a time span of one and a half hours, and 70 to 90 per cent divide over a 40-minute period.

(1) Weight measurements: The cartesian diver balance [18] was adapted for determinations of reduced weight of single amoebae. Reduced weight is defined as the weight of an amoeba immersed in the inorganic amoeba medium. Since the amoebae are weighed in a medium of a density very close to 1.000 (the inorganic amoeba medium), the water contained in the amoebae will not affect its reduced weight

appreciably. Therefore, reduced weight is really the dry weight of the amoeba minus the weight of the volume of water displaced by the dry material.

Incidental techniques for constructing and using the diver balance on a milli-microgram scale are given in [14]. The same diver balance was used for all weight determinations. The equilibrium pressure for the empty diver changed at a constant rate of 11 millimeters per 24 hours. The absolute reduced weight of the smallest amoeba placed on the diver balance, i.e. the individual post-telophase cell, could be determined only to ± 10 per cent owing to difficulties in calibrating the diver balance. However, to define the growth curve of the amoeba it is only necessary to know the relative reduced weight over the cycle, and this could be determined with an accuracy of ± 1 per cent.

(2) Protein measurements: The protein of groups of synchronously growing amoebae was determined by the Folin colorimetric method following specifically the modified procedure described by Lowry *et al.* [6]. A test experiment was carried out with groups of 25, 50, 75, and 100 amoebae taken at random from stock cultures. Over this range the relation between the number of amoebae and the measured protein is linear. The method does not allow an accurate determination of absolute values but the relative amount of protein from point to point of the cell cycle was measurable to ± 7 per cent.

(3) Volume measurements: In order to measure cell and nuclear volumes, amoebae were first flattened to 6 microns in a compression chamber. The areas of flattened surfaces of the nucleus and whole cell were determined with the help of a planimeter, and these values multiplied by the thickness of the preparation to give nuclear and cell volumes. In order to measure nuclear volumes with reasonable accuracy the amoebae had to be squashed to a thickness below 10 microns. Living amoebae rapidly rupture with this treatment and measurements had to be made on fixed cells. The living amoebae were placed in a compression chamber with a small amount of water. A small drop of bromphenol blue-mercury in 95 per cent ethyl alcohol [7] was placed on the underside of a coverslip and the coverslip dropped onto the amoebae. The amoebae were thus flattened, fixed, and stained in the same operation.

The compression chamber was made by applying hydroflouric acid to a small area of an ordinary microscope slide for a few seconds. The details of construction are too extensive to be described here. The resulting depression in the slide was checked for depth and uniformity by compressing small spheres of oil of known volume suspended in distilled water.

The absolute accuracy of the results is limited by two factors; the intrinsic error of the method of measurement itself and the size variation between amoebae in the same growth stage. The former could not be directly checked by repeated measurements on the same amoebae since the amoebae had to be fixed as they were flattened.

Since the edge of the flattened amoeba (in a direction perpendicular to the surface of the depression chamber) is rounded, treating the amoeba as a laminar solid with perfectly flat, perpendicular edges will give a value for volume slightly greater than the true figure. The flattening of the whole amoeba is so severe that this error is negligible. For the nucleus this "edge error" may be as high as 6 per cent for a post-telophase cell and 4 per cent for a pre-prophase cell, giving a relative error of 2 per cent over the life cycle. No corrections have been made in Fig. 4 for this error in nu-

clear volume. The amoebae appeared to be flattened perfectly against both compressing surfaces and error resulting from small folds in the amoebae is felt to be slight if present at all. With regard to nuclear measurements no cytoplasm could be detected either above or below the nucleus. The average nuclear and cytoplasmic volumes determined by measurements on 184 newly divided cells (post-telophases) are given here with standard errors: nuclear volume, $7750\mu^3$ ± 2 per cent; cell volume, $497{,}000\mu^3$ ± 3 per cent.

EXPERIMENTAL RESULTS

Growth in reduced weight.—A dividing amoeba was selected from a stock culture. One of the resulting post-telophase cells was weighed on the diver balance and transferred to a culture dish containing a plentiful supply of *Tetrahymena* and kept at 23°C in the dark. Approximately every 4 hours the growing amoeba was removed from the culture dish and reweighed. The procedure was continued up until division and the last weight measurement made on the dividing cell. Under the above conditions of culture *A. proteus* divides at the end of about 24 hours. In about 15 per cent of the experiments the amoebae failed to divide within 25 hours. Although of interest, the data for these cases were not considered to represent the normal course of cell cycle growth.

Six curves representing the cell cycle growth of six individual amoebae are shown in Fig. 1. Each curve begins with post-telophase cell weight and ends with the weight of the dividing cell. All the curves are essentially of the same character. In each case, growth of the amoeba virtually ceases about four hours before division takes place. Growth from post-telophase to preprophase involves a doubling in reduced weight. An exact doubling must, of course, occur on a statistical basis, but it does not necessarily follow that each amoeba doubles its weight over the course of each cell cycle.

In a few instances the amoebae failed to divide withing 25 hours. The curves in Fig. 2 show the growth of two such amoebae. Here growth has been completed at 20 hours but the cells fail to divide until 18 and 20 hours later. Cells which show this delayed division are not natural variants with characteristically longer life cycles. The two daughter amoebae from the last dividing amoeba in Fig. 2 were cultured, and these divided at 24.5 and 25 hours respectively.

Growth in protein.—Groups of 60 synchronously growing amoebae from different points of the cell cycle were treated with the Folin phenol reagent and optical density measured at 760 millimicrons. Optical density is plotted against time in Fig. 3. Some of the points represent the average of two separate determinations. The point at zero time is for a group of post-telo-

phase cells and the last point is for a group of amoebae entering division. The protein growth curve and the reduced weight growth curves (Fig. 1) follow approximately the same course. While there may be nothing unexpected about this situation, still it adds experimental confirmation to the practice of employing protein increase as a criterion of growth.

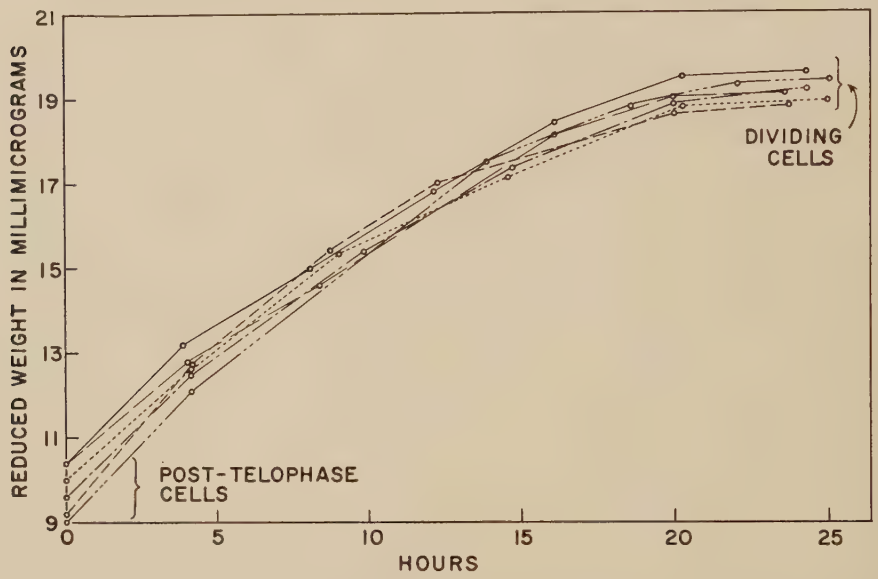

Fig. 1. Growth curves in reduced weight of six individual amoebae from post-telophase to the next division.

Growth in cell and nuclear volumes.— The results of volume studies are plotted in Fig. 4. Cell volume is indicated on the ordinate to the left, nuclear volume is indicated on the right ordinate, and the cytoplasmic/nuclear volume ratio is to be read on the unitless scale to the exteme right. The first point for each curve is the average of 184 measurements and the last point is the average of 120 measurements. The points for interphase volumes of the cell and nucleus are averages of measurements on 14 to 22 synchronously growing amoebae. The growth curve for cell volume roughly parallels the curves for reduced weight and protein increase. This indicates that the density of the amoebae does not vary over the life cycle. Water uptake must be parallel to protein increase and to increase in dry mass.

Growth in nuclear volume is slow until the final 4 hours of the life cycle when the nucleus rapidly attains a volume double that of a post-telophase

cell nucleus. Nuclear volume growth is thus most rapid during the period when growth of the whole cell, as indicated by volume, protein, and reduced weight measurements, has virtually halted. Since the nucleus of *A. proteus* accounts for less than 2 per cent of the total cell volume, changes in nuclear size do not influence the growth curves for the whole cell. Moreover, the increase in nuclear size undoubtedly takes place at the expense of the cytoplasm.

DISCUSSION AND CONCLUSIONS

All but a very few of the growth studies described in the literature concern multicellular systems, i.e. single multicellular organisms or populations of multicellular or unicellular organisms. Such studies are concerned with the increase in numbers of cells of the population, and the results reflect a composite of facts affecting (a) interphase growth, (b) cell division, and (c) interaction within the population and between the population and the environment. The problem under investigation in such a situation is radically different from the problem of the growth of the individual cell over the cell cycle, and it is not surprising that the quantitative relations are different for "growth" in these two very different senses.

The sigmoid character of the population growth curve is not to be expected in the growth curve of the individual amoebae, and if it happened to be found, it would have an entirely different meaning. As a matter of fact, we see in Fig. 1 that it is *not* found. There is no initial lag in the course of growth of an amoeba following division; on the contrary, its initial rate of increase of weight, volume, or protein content is its highest.

During division, there is no growth, a finding that confirms earlier workers, especially Zeuthen [19], and whose interpretation in terms of the nuclear cycle has been discussed in some detail by Mazia and Prescott [8]. Upon termination of division, there is a sharp transition to a period of maximum growth rate. The transition to what I shall call the *predivision period* is not sharply defined, but it is clear that growth in weight, volume, and protein ceases about 4 hours before division. The two principal phases of the life cycle of *A. proteus* at the temperature at which this work was done consists of a growth phase occupying 20 hours following the last division, and a division phase occupying the last 4 hours before division, when there is no growth, and the period taken up by mitosis itself (30 minutes).

The predivision period is of special interest because it constitutes an unknown extending between the completion of growth and the onset of cell division. Its presence would seem to eliminate the consideration that cell

size plays an immediate role in the initiation of cell division. This is emphasized by the amoeba growth curves in Fig. 2 in which the predivision period is abnormally extended. Even amoebae which are prevented from growing to the normal division size by means of controlled starvation will eventually divide, but the cell cycle becomes extended two or three fold depending upon

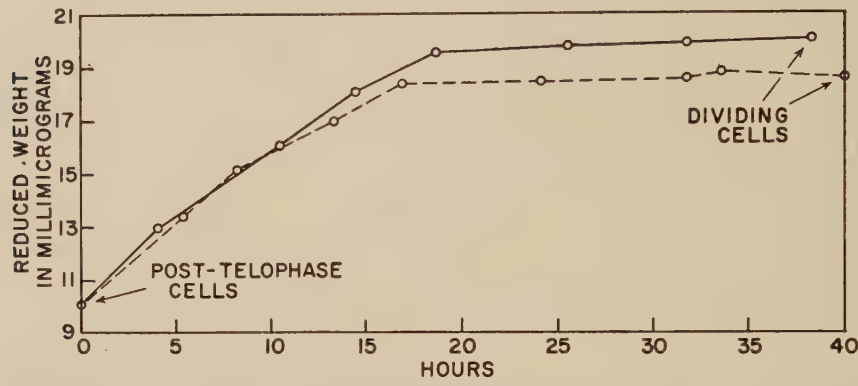

Fig. 2. Growth curves in reduced weight for two individual amoebae the life cycles for which are unexplainably extended. In the normal case division occurs at the end of 24 hours at 23°C.

the severity with which growth has been limited. Therefore, although the *length* of the cell cycle is related to *cell size*, some activity of the cell other than growth acts as the immediate limit to the time of division, and it is logical to suppose that this activity is the basis for the existence of the predivision period.

Not much is known concerning the special activities of a cell just before it enters a recognizable division stage. Zeuthen's studies [19] on the respiration of *Tetrahymena* show a levelling off of respiration shortly before division, indicating a cessation in the synthesis of respiratory machinery and probably, as in *A. proteus*, a general growth stoppage. During division in *A. proteus* phosphate turnover drops by 50 per cent [8] but during the predivision period phosphate turnover does not change significantly from the normal interphase level [12]. If the conclusions drawn in [8] are correct, then it appears that the immediate nuclear contributions to the interphase functions of the cell are maintained during the predivision period. Nevertheless, the rapid volume growth of the nucleus during this period must be a reflection of fundamental changes which possibly are associated directly with the initiation of cell division. Desoxyribonucleic acid or chromosome doubling suggests itself since it is now reasonably certain that desoxyribonucleic acid doubling

does occur during interphase [1, 10, 17]. However, there is good evidence [10] that desoxyribonucleic acid synthesis is completed considerably before the beginning of division.

Since so little is understood concerning the interphase contributions of the nucleus to cell physiology, the changes in the rate of nuclear volume growth

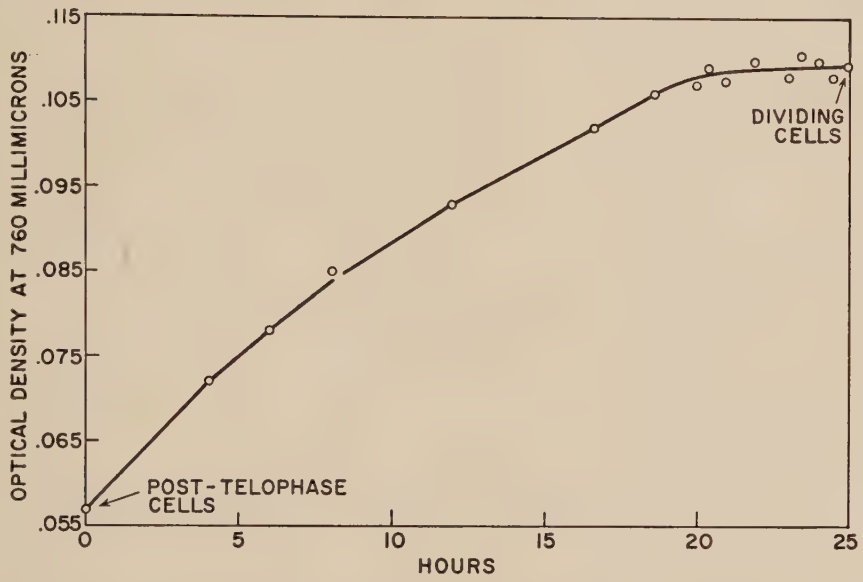

Fig. 3. Growth curve in protein over the complete life cycle. Each point represents a protein determination on a group of amoebae growing in synchrony.

in Fig. 4 are hardly worth further speculation. James [4] has recently presented strong evidence that the nucleus is responsible for maintaining the level of ribonucleic acid in the cytoplasm of *A. proteus* and experiments on the same organism [9] also indicate that part of the protein synthesis of the cell is immediately and directly controlled by the nucleus. Both of these functions, i.e. ribonucleic acid synthesis and nuclear controlled protein synthesis, may well follow characteristic cell cycle patterns. In any case, the rapid nuclear volume growth taking place after cytoplasmic growth has levelled off, strongly suggests that we look to the nucleus for some clue as to the significant activities of the predivision period.

It should be noted that the cytoplasmic/nuclear volume ratio (Fig. 4) is the same just before and after division, rises during the growth period and falls sharply during the predivision period.

With respect to the growth phase for the whole cell the rate of synthetic activity is highest directly following division but falls off slowly until the predivision period is reached. The situation is strikingly similar to that described by Zeuthen [19] for *Tetrahymena*. Here also, growth is not autocatalytic but rather approaches a more linear course. Thus, although growth for a popula-

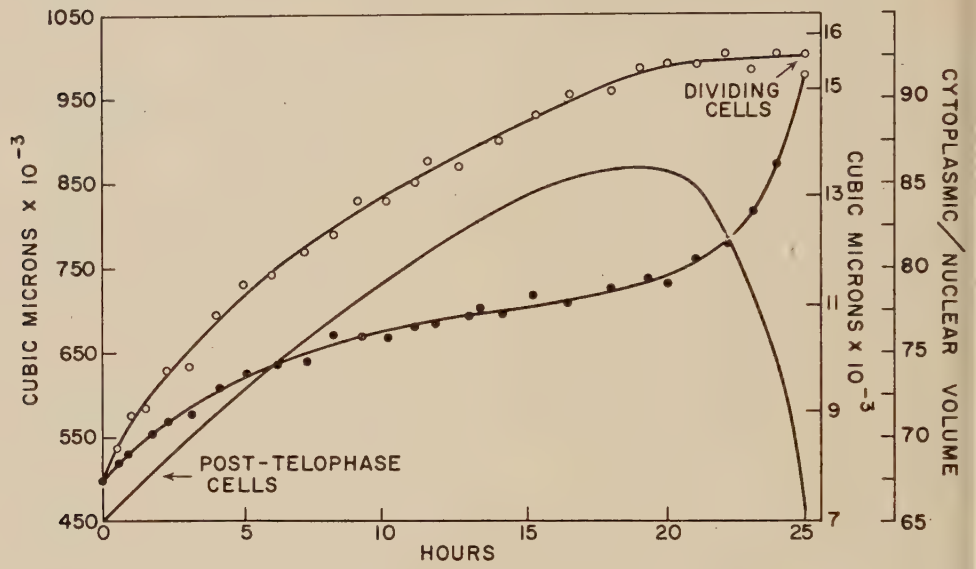

Fig. 4. Cytoplasmic volume, ○; and nuclear volume, ●; and the cytoplasmic/nuclear volume ratio for *Amoeba proteus* from division to division.

tion of cells may be autocatalytic (exponential), the growth of the individual cells in such a population may follow a radically different course. This emphasizes the point brought up earlier concerning the meaninglessness of comparing growth patterns for the two types of systems.

SUMMARY

The growth in weight of single amoebae over the life cycle was measured by means of the cartesian diver balance. Cell growth in volume and protein, and growth in the volume of the nucleus were followed over the cell cycle employing small groups of amoebae in synchronous growth. Growth of the cell is most rapid immediately following division. The rate declines steadily through most of the interphase and virtually ceases during the period of about four hours before division. Nuclear growth is rapid following division but

falls to a lower rate during most of the interphase. During the several hours prior to division, when cell growth has stopped, the nuclear volume increases rapidly.

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ACTION DE LA RIBONUCLÉASE SUR LE DÉVELOPPEMENT EMBRYONNAIRE DES BATRACIENS

I. EXPÉRIENCES DE MICRO-INJECTION

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Dans le but d'étudier le rôle éventuel des enzymes nucléolytiques au cours du développement embryonnaire, nous avons soumis des œufs d'Amphibiens (pleurodèle, axolotl, grenouille rousse) à des micro-injections de diverses préparations enzymatiques (ribonucléase active ou inactivée par oxydation et uréase). Nous avons suivi le développement ultérieur des œufs ainsi traités et étudié, par des méthodes cytochimiques, les modifications intracellulaires.

Avant d'exposer nos résultats, rappelons que Thomas, Rostand et Grégoire [9] ont signalé qu'il est possible de bloquer le développement embryonnaire en piquant des œufs indivis au moyen d'un stylet trempé dans une préparation de ribonucléase. D'autres enzymes, la désoxyribonucléase, la trypsine et la pepsine notamment, ont été également essayés par ces auteurs, sans qu'aucun effet n'ait pu être observé.

Nos résultats confirment ces conclusions tout en précisant que seule la ribonucléase *réduite* agit sur le développement embryonnaire et que cette action dépend donc étroitement des propriétés nucléolytiques de la protéine utilisée.

Le présent travail a pour but de décrire en détails les résultats que nous avons obtenus et qui ont fait déjà l'objet d'une note préliminaire [4].

MATÉRIEL ET MÉTHODE

Nous avons utilisé des œufs d'Amphibens (pleurodèles, axolotls et grenouilles rousses) en voie de segmentation. La ribonucléase (RNase) réduite a été préparée par chromatographie sur amberlite IRC 50 à partir de RNase commerciale (GBI). La fraction D, que l'un de nous a décrite précédemment [3], a été utilisée.

La ribonucléase oxydée, qui a été employée à titre de témoin, a été préparée de deux façons différentes:

1°. Les fractions O, A, B et C, obtenues par chromatographie [3] ont été rassemblées et oxydées en présence d'eau oxygénée dans des conditions douces [2].

2°. Nous avons aussi traité par de l'eau oxygénée la fraction D réduite. C'est cette préparation qui a servi de témoin dans la plupart des expériences décrites ci-dessous.

L'uréase utilisée était un produit Roche.

Les réactifs minéraux étaient tous des produits pour analyse (Merck ou U.C.B.).

Les injections dans les œufs ont été effectuées à l'aide d'un micromanipulateur de Fonbrune, sous une loupe binoculaire, au moyen de micropipettes de verre étiré, de diamètre égal à 0.01 mm environ.

Les volumes utilisés étaient de 0.02 μl. La reproductibilité était de 5 % environ: nous avons pu nous en assurer en injectant des solutions radioactives de radioactivité spécifique connue dans les œufs. Nous avons donc pu déterminer avec précision la quantité d'enzyme introduite dans la cellule; cette quantité sera exprimée, dans le présent travail, en moles/œuf.

Selon le cas, les injections ont été effectuées avant le déganguage des œufs (grenouilles rousse) ou après celui-ci (pleurodèles ou axolotls), de telle sorte que la cicatrisation soit immédiate et les extra-ovats peu importants.

Les œufs ont été fixés au Zenker et des coupes sériées alternes ont été préparées et colorées respectivement à l'Unna et au Feulgen.

RÉSULTATS

Examen Morphologique

Pleurodèles. — Deux cents œufs de pleurodèles ont été injectés. Ces œufs provenaient de 5 pontes différentes. Le tableau ci-dessous résume nos observations. Les stades de la segmentation sont exprimés selon la table de Shumway [5].

TABLEAU I

Etat de l'œuf au moment de l'injection	Quantité injectée (moles/œuf)			Développement max. atteint en 24 h	Pourcentage d'œufs atteignant ce stade
	Uréase	RNase oxydée	RNase réduite		
(2) croissant gris . . .	10^{-10}			(7) 32 blastomères	100
(2) œuf indivis		10^{-12}		(7)	100
(2)			10^{-12}	(2)	100
(2)			10^{-15}	(2)	100
(3) 2 blastomères . . .	10^{-10}			(7)	100
(3)		10^{-12}		(7)	95
(3)			10^{-16}	(4)	10
(3)			10^{-18}	(5) 8 blastomères	20
(3)			10^{-20}	(7)	60
(4) 4 blastomères . . .	10^{-10}			(7)	100
(4)		10^{-12}		(6) 16 blastomères	100
(4)			10^{-17}	(6)	90
(4)			10^{-18}	(7)	100

Remarque: au stade 2 blastomères ((3) dans la nomenclature de Shumway), les œufs ont été injectés dans 1 blastomère seulement.

Tableau II

Etat de l'œuf au moment de l'injection	Quantité injectée (moles/œuf)			Développement max. atteint en 24 h	Pourcentage d'œufs atteignant ce stade
	Uréase	RNase oxydée	RNase réduite		
(2)	10^{-10}			(7)	100
(2)		10^{-12}		(7)	100
(2)			10^{-18}	(3)	30
(3)		10^{-12}		(7)	90
(3)			10^{-15}	(4)	20
(3)			10^{-18}	(5)	50
(3) 2 blastomères . . .	10^{-10}			(8) jeune blastula	100
(3) (1 blast. injecté) .		10^{-12}		(8)	100
(3)			10^{-12}	(3) – (2)	100
(3)			10^{-14}	1/2 (3)	95
(3)				1/2 (8)	
(3)			10^{-15}	1/2 (4)	90
(3)				1/2 (8)	
(4) 4 blastomères . . .	10^{-10}			(8)	100
(2 blastomères		10^{-12}		(8)	100
injectés)			10^{-12}	(3) – (2)	100

Tableau III

Etat de l'œuf au moment de l'injection	Quantité injectée (moles/œuf)			Développement max. atteint en 24 h	Pourcentage d'œufs atteignant ce stade
	Uréase	RNase oxydée	RNase réduite		
(2) croissant gris . . .	10^{-10}			(7) – (8) jeune blastula	100
(2) œuf indivis		10^{-12}		(7) – (8)	95
(2)			10^{-12}	(2)	100
(2)			10^{-14}	(2)	100
(2)			10^{-15}	(2)	100
(2)			10^{-16}	(2)	100
(2)			10^{-17}	(2)	100
(2)			10^{-18}	(3)	20
(2)			10^{-20}	(7)	85

On voit que ni la RNase oxydée, ni l'uréase n'ont d'effet retardateur. La ribonucléase réduite, seule, bloque le développement à des concentrations extrêmement faibles.

Axolotls. — Cent cinquante œufs, provenant de 3 pontes différentes, ont été injectés. Le tableau ci-dessous résume les résultats obtenus.

Les résultats obtenus sont comparables à ceux qui ont été observés sur les Pleurodèles. Ici aussi, la RNase réduite, exerce seule une action inhibitrice nette sur la segmentation.

Grenouilles rousses. — Grâce à la résistance à l'injection particulièrement grande, ces œufs se sont montrés être un matériel de choix. Nous avons injecté environ 500 œufs de grenouilles rousses et nous les avons soumis à un examen morphologique et cytochimique. Le tableau III exprime les résultats donnés par l'examen morphologique extérieur.

Les microphotos 1, 2, 3, 4 et 5 illustrent les résultats des injections effectuées dans les œufs indivis.

Les microphotos 6, 7, 8 et 9 montrent l'action de la ribonucléase oxydée ou réduite quand l'injection est effectuée au stade 2, dans un blastomère seulement.

On voit que les résultats obtenus montrent à nouveau que seule la ribonucléase *réduite* agit sur le développement embryonnaire.

Passons maintenant aux résultats obtenus à l'échelle cytochimique.

Examen cytochimique

Les œufs fixés 12 heures après l'injection présentent parfois des vacuoles précytolytiques. Ces vacuoles se rencontrent surtout dans les œufs injectés de RNase réduite. Il existe donc un net début de cytolyse des œufs lorsque leur développement est arrêté depuis plusieurs heures.

D'une façon générale, tous les œufs traités à la ribonucléase réduite présentent des anomalies nucléaires évidentes : les noyaux sont gonflés et ils contiennent de nombreuses petites masses fortement colorées en rouge à l'Unna; il semble bien s'agir de petits nucléoles. Les œufs traités, au stade deux, par de la RNase réduite concentrée (10^{-14} moles/œuf) montrent des sillons en voie de disparition et une nette dégénérescence des figures achromatiques. Cet effet sur le fuseau et les sillons s'estompe au fur et à mesure que la quantité de RNase injectée diminue. Les morulas partielles obtenues avec des concentrations de RNase inférieures à 10^{-14} moles sont normales et les mitoses sont nombreuses dans la partie qui s'est segmentée; dans l'autre moitié, on trouve un ou plusieurs noyaux gonflés, à chromatine disséminée ou rejetée à la périphérie.

Dans les œufs injectés au stade indivis, on remarque une atténuation des malformations à mesure que la quantité de RNase injectée diminue : les œufs injectés de 10^{-18} moles de RNase se sont divisés, mais les sillons sont en voie de régression; les noyaux sont gonflés et exagérément colorés à la pyronine.

A 10^{-19} moles/œuf, les mitoses sont nombreuses et elles paraissent normales. Les divisions sont cependant moins nombreuses que chez les témoins. A des concentrations plus faibles encore, l'effet est nul.

La figure 10 donne un exemple des anomalies nucléaires observées après coloration des coupes à l'Unna.

DISCUSSION ET CONCLUSIONS

Nous avons donc pu vérifier les résultats de Thomas, Rostand et Grégoire [9] qui avaient observé un blocage des œufs indivis après micro-injection de préparations de ribonucléase relativement pures. Rappelons que, dans ces conditions, les auteurs n'ont observé aucun effet dû à la désoxyribonucléase, la pepsine ou la trypsine.

Nous avons établi, de plus, que seule la ribonucléase réduite a un effet sur la segmentation. Cet effet n'est pas uniquement dû à la présence de groupes sulfhydriles (peu réactifs) au niveau de cet enzyme [2], puisque l'uréase, autre enzyme –SH, n'a pas d'action sur le développement de l'œuf.

Comme la ribonucléase oxydée ne provoque aucun effet, même à des concentrations 10^7 fois supérieures à la concentration effective minimale de RNase réduite, nous pouvons en conclure que l'effet n'est pas dû à la présence de la *protéine* ribonucléase, mais bien à celle de l'*enzyme actif*.

Comme, de plus, les préparations de RNase utilisées présentaient toutes les garanties de pureté désirables, l'action observée ne peut être due à la présence d'une impureté quelconque : une telle impureté serait, d'ailleurs, au concentrations faibles utilisées, à coup sûr inoffensive.

Il est évident que l'interprétation actuelle des phénomènes observés n'est guère facile : il ressort de l'examen cytochimique que la ribonucléase exerce une action semblable à celle décrite par l'un de nous dans le cas du dinitrophénol [1] (arrêt de la division, gonflement des noyaux); elle agit, cependant, à des concentrations incomparablement plus faibles. Il se peut toutefois que cette analogie soit purement fortuite et il faudrait établir si la RNase entrave, comme le dinitrophénol, les phosphorylations oxydatives avant de conclure. Il est difficile d'imaginer en quoi consiste l'action de l'enzyme à des concentrations aussi faibles : *in vitro*, de telles doses n'auraient,

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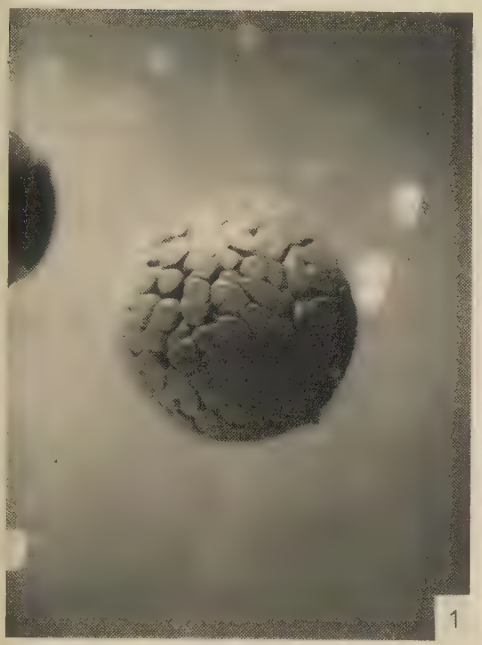
Fig. 1. Œuf injecté au stade indivis, de 10^{-12} moles de ribonucléase oxydée.
Fig. 2. Œuf injecté au stade indivis, de 10^{-10} moles d'uréase.
Fig. 3. Œuf injecté au stade indivis, de 10^{-12} moles de ribonucléase réduite.
Fig. 4. Œuf injecté au stade indivis de 10^{-18} moles de ribonucléase réduite.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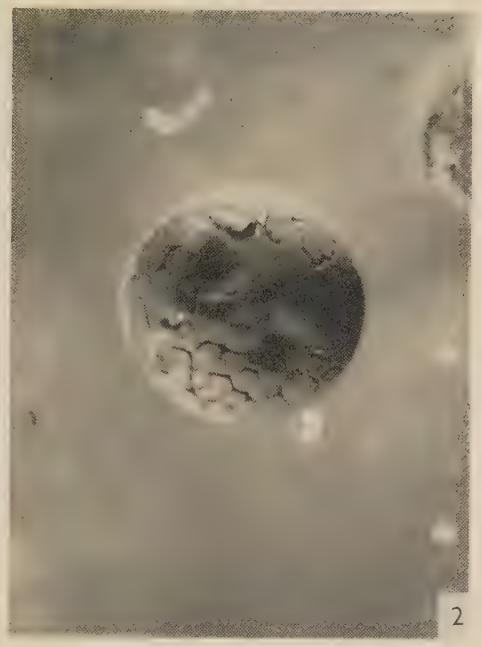
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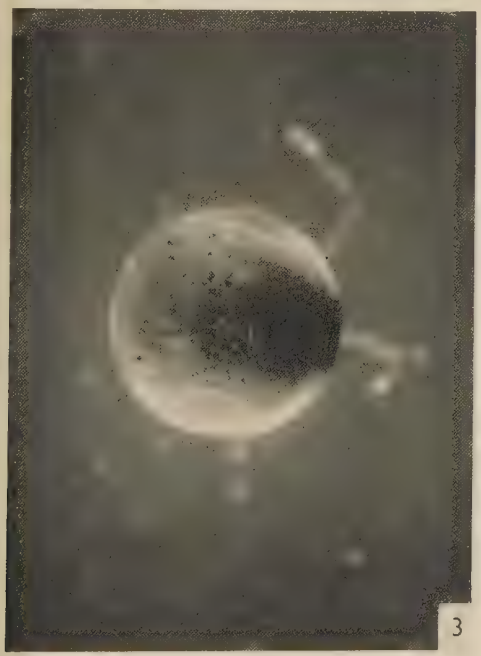
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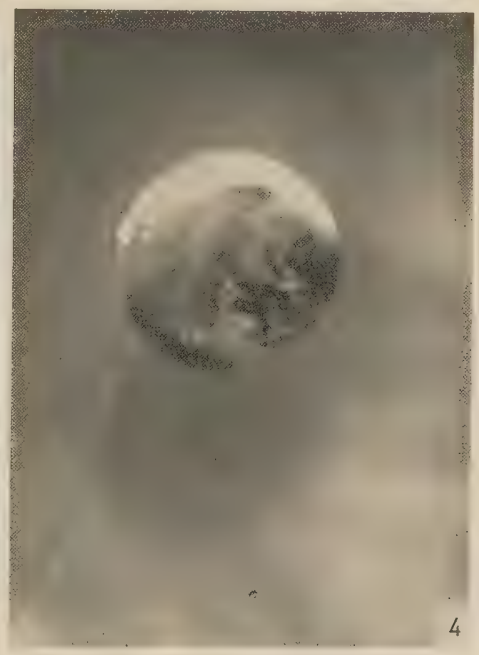
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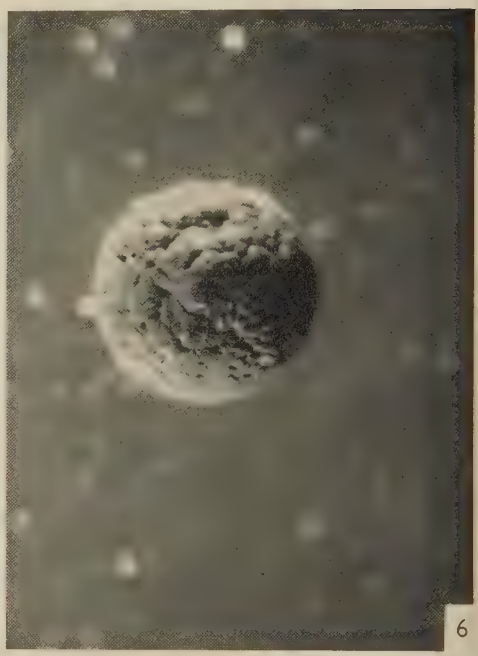
5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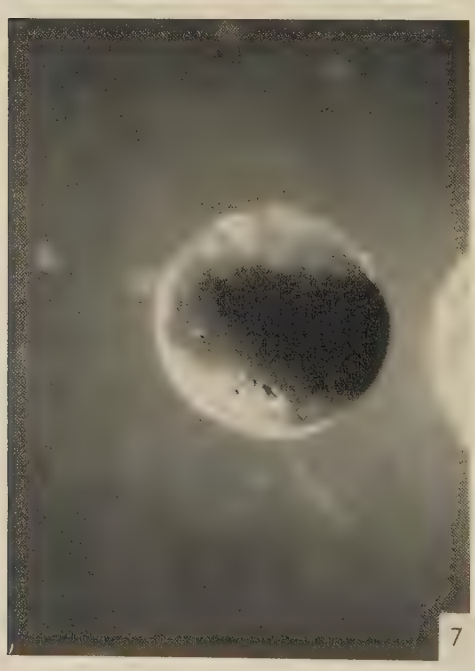
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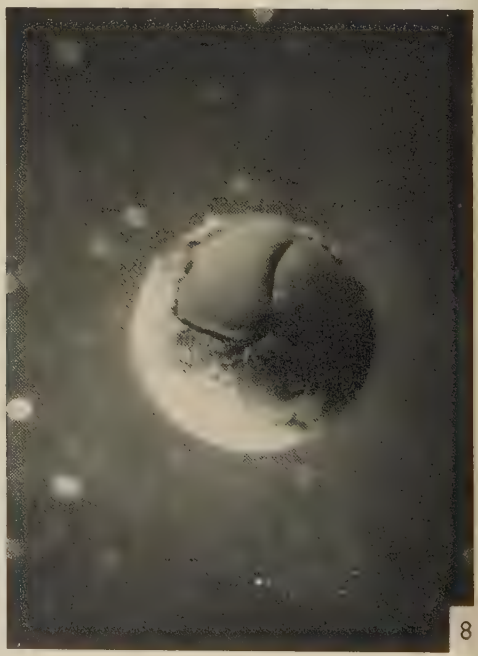
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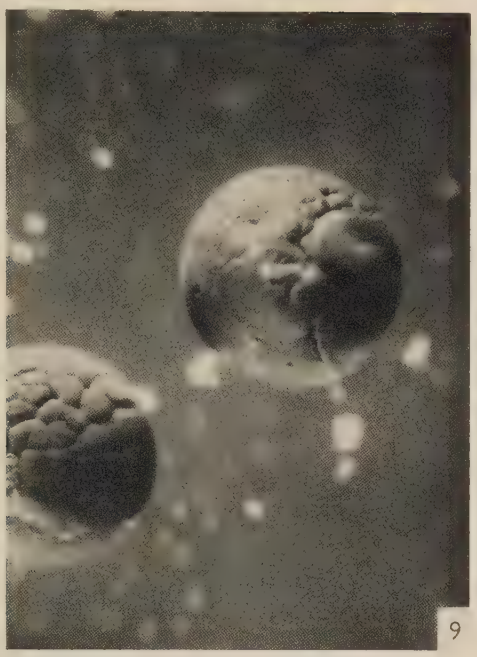

Fig. 9. Œuf injecté au stade 2 blastomères, de 10^{-15} moles de ribonucléase réduite.

en effet, pas d'effet appréciable sur la dépolymérisation de l'ARN. Dans ces conditions, on peut se demander si la présence de traces de ribonucléase dans l'organisme aux premiers stades de la segmentation ne catalyserait pas la libération intracellulaire de substances toxiques pour le développement ultérieur. On ne peut dire actuellement si ces substances toxiques sont constituées par d'autres enzymes ou par de la ribonucléase libérée à partir d'un précurseur (ou à partir d'un complexe nucléoprotéique préexistant). Des expériences récentes de Hultin et Lundblad [6] et de Roth [7] ont, en effet, montré que la RNase peut libérer ou activer, dans des homogénats ou des poudres sèches d'œufs, des enzymes hydrolytiques tels que des protéases ou de la désoxyribonucléase.

Il est donc probable que l'injection de RNase met en route toute une chaîne de réactions chimiques et biologiques. Il se pourrait aussi que les molécules de la ribonucléase injectée se fixent sélectivement sur un centre

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Fig. 5. Œuf injecté au stade indivis, de 10^{-20} moles de ribonucléase réduite.
Fig. 6. Œuf injecté au stade 2 blastomères, de 10^{-12} moles de ribonucléase oxydée.
Fig. 7. Œuf injecté au stade 2 blastomères, de 10^{-12} moles de ribonucléase réduite.
Fig. 8. Œuf injecté au stade 2 blastomères, de 10^{-14} moles de ribonucléase réduite.

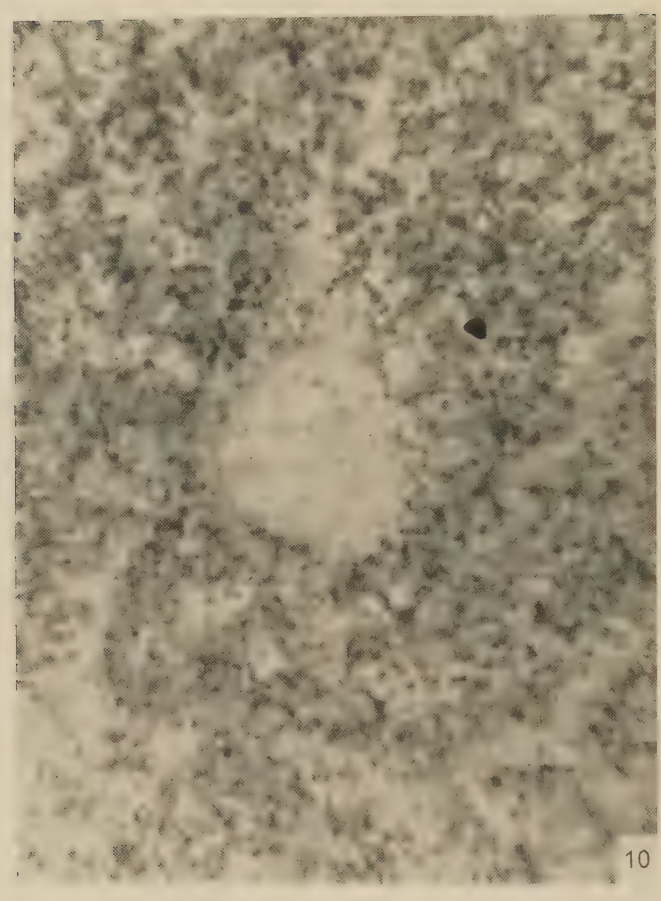

Fig. 10. Coupe (colorée à l'Unna) montrant l'action de la ribonucléase sur le noyau de l'œuf.

actif (membrane cellulaire ou fuseau, par exemple) et que cette fixation suffise à provoquer le blocage de la division.

Des expériences effectuées à l'aide de préparations de ribonucléase marquée par de l'iode radioactif [5] montent que la ribonucléase réduite se fixe sélectivement sur les mitochondries. La ribonucléase oxydée ne présente, au contraire, pas de lieu de fixation défini.

On peut dès lors imaginer que la forme réduite de l'enzyme, en s'accolant aux mitochondries (par des groupes non disponibles dans le cas de la forme oxydée) empêche ces dernières de remplir leurs fonctions normales et de ce fait paralyse les processus mitotiques : il en résulterait, par exemple, une inhibition du couplage entre les phosphorylations et les oxydations, ce qui

expliquerait la similitude des effets cytologiques et cytochimiques provoqués par la RNase réduite et par le dinitrophénol.

Des recherches ultérieures sont, de toute évidence, nécessaires pour élucider ces questions; mais, de toute façon, il est hors de doute que la ribonucléase, une fois introduite dans la cellule, constitue un inhibiteur très puissant de l'activité mitotique, même à des doses extrêmement faibles.

RÉSUMÉ

Des œufs d'amphibiens en voie de segmentation, ont été soumis à des micro-injections d'uréase, de ribonucléase active ou inactivée par oxydation. La ribonucléase réduite, seule, a montré une action inhibitrice nette sur la segmentation, à des concentrations de l'ordre de 10^{-15} moles par œuf.

L'examen cytochimique des embryons bloqués a révélé un gonflement des noyaux, chargés de petites masses fortement colorables à la pyronine; les œufs traités au stade 2 blastomères présentaient une régression des sillons et une dégénérescence des figures achromatiques.

Ni la ribonucléase à l'état oxydé, ni l'uréase, autre enzyme à groupe –SH n'ont inhibé les divisions cellulaires : la ribonucléase, sous sa forme *enzyme actif*, est donc responsable du blocage de la segmentation.

Le rôle joué par la ribonucléase au cours des phénomènes observés, est discuté.

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BRIEF NOTES

A COMPONENT PROMOTING OXIDATIVE PHOSPHORYLATION, RELEASED FROM MITOCHONDRIA DURING AGING

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The reconstruction of oxidative phosphorylation in the mitochondrial succinic oxidase system aged in Mg^{++}-free media in the presence of yeast hexokinase has been the subject of a recent report from this laboratory [2]. In this system, adenosine triphosphate (ATP) is the only addition required for a restoration of the phosphorylative ability, its effect being potentiated by Mn^{++}, or by relatively high concentrations of Mg^{++}. In a later communication [1], the disappearance of the phosphorylative ability upon aging, and the reversal of this effect by the addition of ATP and Mn^{++}, could be correlated with reversible changes in the physical state of the mitochondria, as revealed by optical density measurements. On the basis of these findings the hypothesis has been formulated [2] that ATP, bound to the mitochondrial structure by means of either of the above cations, functions as a cofactor responsible for coordinating the phosphorylative mechanism with the respiratory system.

It was observed in the course of this investigation that, on prolonged preincubation, the aged suspensions lose their responsiveness to added ATP. It was therefore considered that, during the process of aging, other components involved in oxidative phosphorylation may also be released from the mitochondria, although not necessarily depleted from the medium with the same rapidity as is ATP (due to the presence of hexokinase). Experimental support for this idea has been obtained by comparing the effects of added ATP and Mn^{++} on mitochondrial suspensions aged for relatively short time periods with those on mitochondria re-isolated from these suspensions and incubated in a new incubation mixture.

Rat liver mitochondria were prepared in 0.25*M* sucrose and suspended in a Mg^{++}-free, isotonic KCl-sucrose medium containing orthophosphate, adenylic acid, glucose and hexokinase, as described previously [2]. Suspensions of 30 ml, containing mitochondria from one rat liver, were aged for 10 minutes at 30°C under shaking in a 200 ml Erlenmeyer flask, and then cooled rapidly to 0°C (at this low temperature the aging process was found to be decelerated by about 6–8 times). The suspension was divided into two parts and both were centrifuged at 0°C in the high speed attachment of an International Refrigerated Centrifuge for 5 minutes at 5000 *g*. Of the two mitochondrial pellets so obtained (containing about 87 per cent of the protein-N of the original suspension), one was resuspended in its own supernatant, while in the other case the supernatant was discarded and replaced by an equal volume of a new incubation mixture of the same composition as the original. Aliquots of both suspensions were supplemented with Mg^{++} and incubated in the presence of succinate. Respiration and phosphorylation were measured as described previously [2].

The results of a typical experiment are shown in Table I. From these it emerges that the ATP-Mn^{++}-induced phosphorylation is significantly higher in the presence than in the absence of the aging medium. This difference cannot entirely be ascribed to a removal of cytochrome *c*, since addition of the latter results in but a partial stimulation of the rates of respiration and phosphorylation. The fact that increasing concentrations of cytochrome *c* yield fairly constant phosphorylation values precludes also the possibility that cytochrome *c*, while promoting respiration, causes a decrease of the actual rate of phosphorylation, as was recently shown by Lehninger *et al.* [3] to be the case with ascorbic acid as a substrate. It is therefore concluded that the preincubation medium contains a component, released from the mitochondria upon aging, and not identical with cytochrome *c*, which has the ability to promote oxidative phosphorylation in the ATP-Mn^{++}-restored mitochondrial succinic oxidase system. A closer characterization of this component is the subject of further study.

TABLE I

Effect of the aging medium on oxidative phosphorylation in reconstructed mitochondrial systems.

Each Warburg vessel contained: KCl, 150 μmoles; orthophosphate, 50 μmoles; adenylic acid, 4.3 μmoles; glucose, 60 μmoles; sucrose, 125 μmoles; succinate, 30 μmoles; Mg^{++}, 4 μmoles; hexokinase, 5fold excess [2]; mitochondria, 0.3 g rat liver. Additions (where indicated): ATP, 1.5 μmoles; Mn^{++}, 1 μmole. Final volume, 2 ml. Gas phase, air. Temp. 30°C. Time of incubation, 10 min.

System	Cytochrome *c* *M*	*Without* ATP and Mn^{++}		*With* ATP and Mn^{++}	
		Respiration μatoms O	Phosphorylation μmoles P	Respiration μatoms O	Phosphorylation μmoles P
Fresh mitochondria . .	—	9.9	17.9		
Aged mitochondria					
Aging medium present	—	5.5	1.1	9.3	9.9
	1.2×10^{-6}	7.1	1.1	10.0	9.6
	6.0×10^{-6}	6.4	1.0	10.2	9.1
	3.0×10^{-5}	6.5	1.0	10.9	9.1
Aging medium absent	—	3.2	0.2	4.6	1.5
	1.2×10^{-6}	3.7	0.3	3.7	4.5
	6.0×10^{-6}	2.9	0.2	5.8	3.8
	3.0×10^{-5}	4.3	0.2	6.6	4.2

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SOME EFFECTS OF COLCHICINE ON *CHLAMYDOMONAS CHLAMYDOGAMA* BOLD

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The more or less typical effects of colchicine upon plant cells have been summarized by Cornman [2] and Eigsti and Dustin [3]. These include abnormally large and misshapen cells, polyploidy, and various nuclear deformities, i.e., bridged, lobed, and fragmented nuclei. Gavaudan and Kobozieff [4] subjected an unidentified species of *Chlamydomonas* to 0.1 per cent colchicine for 48 hrs and subsequently observed abnormalities in size and number of daughter cells, pyrenoids, and nuclei. Vandenvries and Gavaudan [5], on the other hand, reported no cellular anomalies in various members of the same genus treated with the alkaloid. Cornman [2] added colchicine in amounts varying from 0.005 to 0.015 per cent to a basal culture medium inoculated with *Chlamydomonas pseudococcum* Lucksh. No cytological aberrancies were observed and the author concluded that the organism, although retarded somewhat in growth, was cytologically resistant to colchicine. The present investigation shows that the green alga, *Chlamydomonas chlamydogama* Bold, grown on a culture medium containing 0.2 per cent colchicine, develops marked and obvious abnormalities.

The algae were spread over the surface of inorganic salt agar plates [1] to which 0.2 per cent filtered-sterile colchicine had been added before the plates were poured. Preliminary experiments had demonstrated that 0.2 per cent colchicine is just sublethal for the organism. It was observed also that autoclaving, either with the basal medium or separately, partially impaired the action of the drug. The organisms grew rapidly on this medium, but abnormal increase in size and variations in morphology were observed at the end of 48 hrs incubation at 22°C and illumination at 100 foot candles for 12 hrs daily. The deviations became more marked in older cultures. Measurements of cells grown in colchicine-containing media (both liquid and solid) were compared with those of cells grown in the absence of colchicine. After 8 weeks, 150 cells from each of the media were measured with a filar micrometer. The measurements indicate that colchicine added to both liquid and solid media causes an average increase of $\frac{1}{3}$ to $\frac{1}{2}$ in cell size and definite changes in cell shape. Photomicrographs of cells grown on agar media were made after 16 days. Fig. 1A illustrates normal cells on inorganic salt agar, while Fig. 1B illustrates the abnormally large size, irregular shapes and other aberrancies present in most of the individual cells grown on colchicine-containing agar. Camera lucida drawings show an increase in the number of stigmata and pyrenoids (Fig. 2C, E). The nuclei are larger than normal (cf. Fig. 2A and 2F) and the chloroplasts are distorted in some individuals. Preparations stained with Haidenhain's iron-hematoxylin or treated by the Feulgen method contained cells with greatly enlarged nuclei and, in many instances, several nuclei which some-

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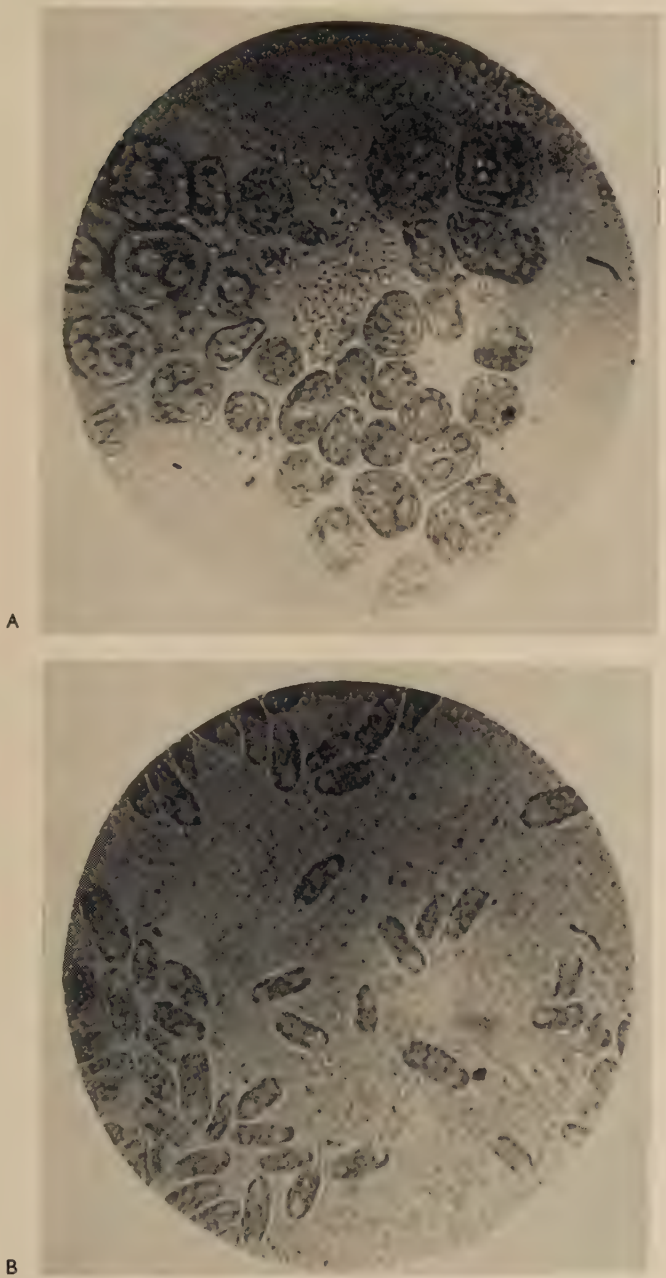

Fig. 1. Photomicrographs of vegetative cells of *C. chlamydogama* after 16 days on inorganic salt agar (*A*) and on the same medium containing 0.2 per cent colchicine (*B*). ×660.

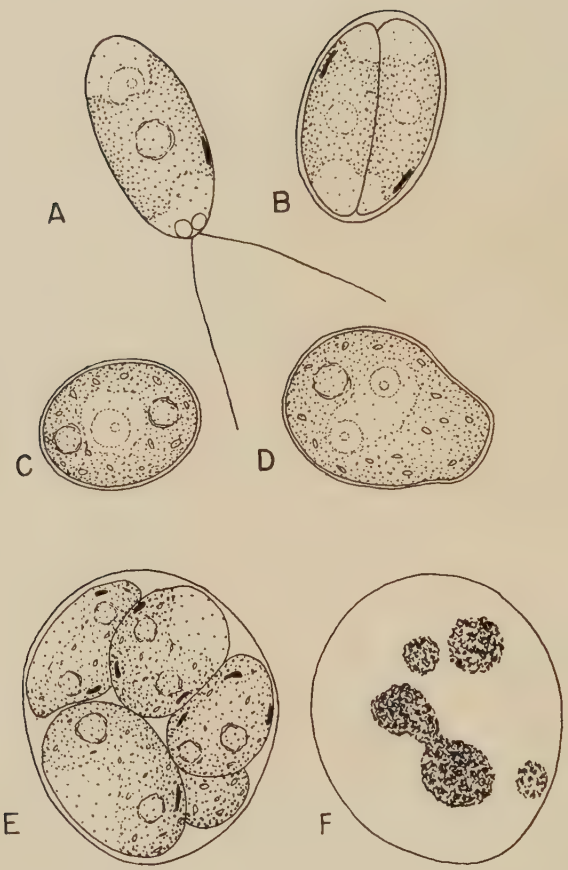

Fig. 2. Camera lucida drawings of cells of *C. chlamydogama* on inorganic salt agar (*A*, *B*) and on the same medium containing 0.2 per cent colchicine (*C*–*F*): *C*, cell with two pyrenoids; *D*, binucleate, misshapen cell; *E*, giant cell containing five unequally-sized daughter cells—note numerous stigmata and multiplication of pyrenoids; *F*, giant cell, Feulgen preparation. × 1070.

times were connected by Feulgen-positive bridges (Fig. 2D, F). In several prophase figures, in which the chromosomes could be distinguished, it was observed clearly that they exceeded the number of chromosomes of the untreated cells, although exact counts were not feasible. These phenomena suggest that the increase in cell size is probably caused by the successive arrest of mitoses and repeated formation of restitution nuclei. Daughter cells, usually uniform in size and in number, were variable in colchicine-treated cultures (cf. Fig. 2B and 2E). The irregularities observed in cell reproduction are a further manifestation of cytological disturbance.

When giant cells were transferred to liquid medium containing no colchicine, they gave rise to *apparently* normal cells, for after a few divisions, a majority of the cells in such a culture regained their normal shape and appearance and became actively motile. No persistent giant strains could be established. Abnormal cells transferred to fresh medium containing colchicine gave rise to progeny of aberrant sizes and shapes. The abnormalities cannot be attributed, therefore, to a depletion of water and/or nutrients.

These preliminary observations suggested that further cytological and genetical study of the colchicine effect on *Chlamydomonas* would be fruitful, and comparative studies of several species are in progress.

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THE TAURINE CONCENTRATION OF DEVELOPING CHICK EMBRYO[1]

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Machlin, Pearson and Denton [8] have recently reported that sulfate-S^{35} is incorporated into taurine in the chick embryo. The same results were obtained by Lowe and Roberts [7]. In our laboratory we have been interested in the mechanism of taurine formation and in the elucidation of the probable role of taurine in the organism. A survey of the taurine concentration of organs from several animal species was recently presented [2]. This survey has now been extended to the chick embryo.

Materials and methods.—Eggs from crossings of White Leghorn and Rhode Island chickens were obtained from the Poultry Plant of Texas A. and M. College. The eggs were incubated in a commercial incubator at about 37.5°C. After various periods of incubation, eggs were taken out and the embryos pooled for analysis. The number required for an analysis depended on the stage of development. In the late stages, one sufficed, but in the early stages many embryos had to be pooled in order to have sufficient material for the analysis.

Analyses were carried out in whole embryos and in isolated embryo hearts. This organ was chosen because in the adult animal the heart has the highest concentration of taurine of all organs analyzed. This applies to the other species as well as the chick. The hearts from several embryos were pooled to obtain sufficient material for analysis. The analytical procedure used was based on paper chromatography and separation of taurine from other ninhidrin-reactive substances by means of a cation exchange resin. The tissues were first extracted by the alcohol-chloroform method reported previously [1]. The protein and fat-free extract was then used for the analysis. The heart extracts were analyzed by two-dimensional chromatography using phenol–water and 2,4-lutidine–water as solvents. The quantitation of the spots was

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carried out according to a method described previously [6]. The whole embryo extracts were fractionated by means of Dowex-50 (8 per cent cross linkage, 200–400 mesh) in the hydrogen form. The extract was passed through a 4 × 1 cm column of this resin. Taurine passed through and the first 5 cc contained all the taurine. For final analysis the eluate was concentrated and chromatography on filter paper carried out (one dimensional chromatography with lutidine gave excellent separation from the few ninhydrin-reactive substances that pass with the taurine).

TABLE I

Taurine content of whole chich embryos.

Age of embryo in days	1	2	3	4	5	6	7	10
μM taurine per 100 mg dry protein	0.25	1.21	4.92	9.72	9.70	9.87	9.56	9.30

TABLE II

Taurine content of isolated chick embryo hearts.

Age of embryo in days	7	11	13	14	16	New hatched	Adults
μM taurine per 1 g tissue*	4.4	6.2	6.9	8.9	11.2	19.0	18.4

* Wet weight.

Results and Discussion.—Eighteen- to 20-hour chick blastoderms [5] and yolk and albumin [3] of unincubated chicken eggs contain no detectable taurine.

In the present experiments with whole embryos (Table I) we found definite spots corresponding to taurine in chromatograms of embryos of all stages studied (24-hour through 11-day). The taurine content of the embryos goes up rapidly through the 3rd day of incubation and levels off on the 4th day. Thereafter the level of taurine remains fairly constant.

In the isolated hearts (Table II) the concentration of taurine goes up steadily from the 7th day through hatching. Thus, after the taurine concentration had reached a plateau in the whole embryo, it was increasing in the isolated heart.

It is possible that whereas the taurine content of the heart was increasing, tissues other than the heart were being depleted of taurine. Supporting this possibility is the finding [10] that fetal mouse brains have more taurine than adult brains. Furthermore, in chromatograms of embryonic chick brain, we have found that taurine decreases from a level of 6.4 to about 5.5 micromoles/gm wet weight between the 12th day of incubation and hatching. In the mosquito, *C. quinquefasciatus*, the level of taurine goes down with development between the larval and pupal stages [9]. On the other hand, Gordon [4] found an increase in taurine between embryonic calf muscle and that of the adult.

Another way whereby the taurine concentration in the entire embryo could be kept constant while that of the heart rises, is by differential growth of tissues of high

and low taurine content. Thus, a rise in taurine in certain tissues would be balanced by a greater growth rate of tissues of low taurine content. Either or both of the possibilities could contribute to the maintenance of a constant level of taurine in the entire embryo.

In summary: (1) taurine is present in low concentration in chick embryos at the end of 24 hours of incubation; (2) it rises rapidly through the 4th day at which time it levels off and remains more or less constant through the 10th day of incubation; (3) taurine concentration in the heart increases from the 7th day through hatching.

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BUFFER EFFECT IN FERMENTATION SOLUTIONS

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In fermentation chemistry, as well as in all biochemical research, it has become a practice during the last decades to buffer solutions to a definite pH. The buffering has in general been effected by known buffer systems aiming at a definite pH, often with no regard to the composition of the buffer. However, already Hägglund [8] has drawn attention to the different effect of various buffer systems on the fermentation of sugars by yeast, and in particular to the exceptional trend of the pH curve of fermentation with acetic acid–acetate buffer, and he has suggested that an explanation for the phenomenon might be found in cell permeability.

In the recent years the permeability of microbe cells has been frequently discussed in different connections. Attempts have been made to explain speculatively the experimental results, otherwise uninterpretable, on the basis of the permeability or nonpermeability of cells. In a few rare cases only, the claims have had experimental support (cf. [4] and especially [7]). The vital staining method introduced by Collander [6] seems to afford a more convincing proof for the permeability of microbe cells. This method makes it possible to follow the penetration of a basic substance into the

cell by observing the changes in the colour of the indicator, neutral red, within the cell. Äyräpää [1] has later employed this method in his studies on the base permeability of yeast cell. We have applied the neutral red staining in order to elucidate the buffering effect of alkaline buffer systems on yeast suspensions.

Equal amounts of a 0.02 per cent aqueous solution of neutral red and the buffer solution to be examined were added to a yeast suspension containing 20 g freshly washed brewer's or baker's yeast (dry matter about 25 per cent) per 100 ml tap water. A number of different basic buffer systems were taken for comparison. In order to include a suitable selection of buffers, pH 10 was chosen for the pH value [11]; the phenomenon was discernible also at lower pH values. The experiment comprised: (a) glycocoll–NaOH buffer according to Sørensen, (b) borate–NaOH buffer according to Sørensen, (c) ammonia–ammonium chloride buffer according to Michaelis, (d) boric acid–soda buffer according to Atkins and Pantin, (e) soda–bicarbonate buffer according to Menzel.

The result was dramatically clear. Of all the buffers examined, only the ammonia–ammonium chloride buffer of Michaelis turned the red-dyed cell mass to yellow, thus indicating that the effect of the buffer had reached the inside of the cells. The change was instantaneous and lasting, the colour remained yellow for almost 24 hours; the reappearance of the red tinge in the course of 24 hours proved that the cells had survived, an observation which could be confirmed both microscopically, with methylene blue, and by fermentation test. Hence, the change of the reaction within the cell by the ammonia–ammonium chloride buffer and, of all the buffers tested, by that alone, was clearly perceptible. All the other buffers were able to buffer only the external solution. The potassium salts behaved in the same way as the sodium salts.

Unfortunately, the vital staining method could not be applied to an elucidation of the cell permeability in regard to acid buffer systems. At least we did not succeed, despite all efforts—a great number of both ordinary and fluorescence indicators were tested in finding a single vital stain suitable for the purpose. Consequently we proceeded to test the suitability of the titration method first with organic acids, then with buffer systems.

The experiments with acids were carried out at room temperature by adding 0.2*N* acid solution to an equal volume of yeast suspension prepared from freshly washed and filtered brewer's or baker's yeast in a ratio of 100 g fresh yeast to 100 ml tap water. The cell mass in this case takes up a part of the volume of the suspension, while the cell interspace water of the yeast cake increases the volume of the dilution water. When the dry matter content of the yeast cake is 25 per cent the cell interspace water forms 28.6 per cent of its volume, and when the dry matter content is 23 per cent the cell interspace water is 34.3 per cent of the volume [9, 13]. Hence, if—as in the following experiment with baker's yeast—100 g yeast (dry matter 25.0 per cent), corresponding to about 92 ml, is suspended in 100 ml of water, the volume of external water of cells is, in 100 ml of suspension, 65.8 ml, 34.2 ml of the volume being occupied by the cell mass. On addition of 100 ml of 0.2*N* acid solution to 100 ml of suspension, the acid solution becomes diluted with 65.8 ml water, provided that the acid does not at all penetrate into the cells; in this way the estimated acid concentration is obtained. The results recorded in the tables were calculated on the basis of this value. The titrations were made in the water phase separated from the suspension by 10 min.

centrifugation (3000 rpm.) using thymolblue-phenolphthalein as an indicator. The results in regard to some organic acids are shown in Table I.

The results indicate that the free fatty acids penetrate rapidly into the yeast cells. Citric and tartaric acids are practically unable to penetrate into the cells and even succinic acid penetrates very slowly. Lactic acid represents an intermediate stage.

TABLE I

Penetration of organic acids into yeast cells reflected by the lowering of the acid content of the external solution.

Excluding the volume taken up by the cells, the calculated acid concentration of the external solution immediately after the acid addition should have been 0.061*N* in the experiment series with brewer's yeast and 0.060*N* with baker's yeast.

Acid added	Acid concentration of the water phase of the suspension in per cent of the calculated (the volume of the cell mass substracted)					
	Brewer's yeast (dry matter 25.8 per cent)			Baker's yeast (dry matter 25.0 per cent)		
	"Immediately"	30 min.	60 min.	"Immediately"	30 min.	60 min.
Acetic acid	70.7*	70.3	70.1	75.4	75.0	74.8
Propionic acid	71.9	71.1	70.1	76.4	76.4	76.5
Butyric acid	71.1	70.6	70.6	75.8	75.8	76.0
Lactic acid	96.3	90.2	86.4	97.7	96.2	95.2
Succinic acid	95.1	92.5	90.2	99.1	98.7	98.2
Tartaric acid	98.1	98.1	97.1	99.6	99.6	99.4
Citric acid	97.2	97.4	96.5	99.9	100.0	100.0

* Acetic acid and lower fatty acids in general penetrate into the cell so rapidly (cf. e.g. [5]) that observation of the estimated acid concentration was not even possible by the technique used.

Buffers containing organic acids behave correspondingly, provided that free acid is more liberally present. The acid buffers were prepared by adding to a 0.2*N* acid solution its sodium salt solution of corresponding concentration in a ratio of 1:1. The experiments were performed as before by mixing equal amounts of yeast suspension and the following buffers (cf. [11]): (a) acetic acid–acetate buffer according to Walpole, in principle as Michaelis's acetic acid–sodium acetate buffer, (b) lactic acid–sodium lactate buffer, in principle according to Michaelis, (c) succinic acid–sodium succinate buffer, in principle as the succinate–succinic acid buffer used by Runnström and Brandt [2], (d) citric acid–sodium citrate buffer, in principle as the citrate–NaOH buffers of Sørensen and of Kolthoff & Vleeschhouwer or the citrate–citric acid buffer of the latter ones. In order to check that the results of the experiments performed according to the entirely different methods with basic buffer systems would be comparable, the titrations were also made in solutions buffered with earlier tested borate–NaOH and ammonia–ammonium chloride. For the sake of analogy, they were in this case prepared by mixing 0.2*M* basal solutions in sufficient amounts to attain pH 10.

TABLE II

Penetration of the acid or base components of buffer solutions into the yeast cells, reflected by the lowering of the acid/base content of the external solution.
Excluding the volume taken up by the cells, the calculated acid/base concentration of the external solution immediately after the buffer addition should have been, in the experiment series with brewer's yeast, 60.8 per cent, and with baker's yeast 60.4 per cent of the acid/base concentration of the initial buffer solution.

Buffer solution	The acid/base concentration of the water phase of the suspension in per cent of the calculated (the volume of the cell mass substracted)									
	Brewer's yeast (dry matter 25.7 per cent)					Baker's yeast (dry matter 25.3 per cent)				
	"Immediately"	30 min.	1 h	2 h	6 h	"Immediately"	30 min.	1 h	2 h	6 h
Acetic acid–Na-acetate . . . . pH 4.7	68.1*	67.5	67.7	67.8	64.9	69.6	69.6	69.4	70.6	73.6**
Lactic acid–Na-lactate . . . . pH 3.7	92.7	85.3	79.5	75.3	70.8	98.3	96.5	94.0	90.2	82.9
Succinic acid–Na-succinate . . . . pH 4.7	99.9	97.9	98.0	92.6	84.7	99.9	100.0	99.9	100.0	99.7
Citric acid-Na-citrate . . . . . pH 4.4	99.4	99.4	98.2	98.3	97.6	99.8	99.8	99.9	99.5	99.3
Ammonia–ammonium chloride pH 10.0	54.8*	54.0	52.9	50.9	49.2	56.8	56.1	56.1	54.6	54.6
Borate–Na-hydroxide . . . pH 10.0	90.4	88.0	87.1	83.8	81.3	97.7	97.7	96.8	97.7	95.1

* In regard to the penetration velocity of acetic acid cf. Table I, footnote; what there is said holds good for the penetration velocity of ammonia (cf. [5]).

** In experiments with baker's yeast, the increase in the acid content of the solution was a rule in the later stages when acetic acid buffer was used; the occurrence of succinic acid in the solution could be shown by paper chromatography (cf. [3]).

The titrations were made by using thymolblue–phenolphthalein or bromcresol green–methyl red mixed indicators. The results are recorded in Table II.

The results indicate in full conformity with those obtained with organic acids that of the buffering systems tested only the acid component of the acetic acid–acetate buffer penetrates rapidly into the cells. The acid component of lactic acid–lactate buffer penetrates but slowly into the cells, that of succinic acid–succinate buffer very slowly, hardly at all with baker's yeast. Citric acid–citrate buffer buffers only the external solution. The results obtained by using basic buffer systems confirm fully the findings made according to the vital staining method: in the ammonia–ammonium chloride system ammonia penetrates rapidly into the cells, while the borate–NaOH buffer buffers in the first place only the external solution.

In order to obtain direct evidence of the fact that acetic acid—contrary to e.g. citric acid—really penetrates into the cell, the cells, previously suspended in the acid

solution, filtered and washed, were disintegrated by liquid air. 10 g of newly washed baker's yeast was suspended in 10 ml of 0.2*N* acetic or citric acid or water. The cell mass was separated from the solution sharply employing a Büchner funnel, washed rapidly with 10 ml of water and freezed in liquid air. After the temperature of the mass transferred into the room temperature had risen to 0°C it was completely melted by adding 10 ml of water about 80°C temperature. Instead of titration of the acid content, the pH of the plasmolysate was measured potentiometrically and the following results were obtained: Yeast treated with acetid acid: pH 4.95; yeast treated with citric acid: pH 5.75; and yeast treated with water: pH 5.85.

In a few occasional instances only the idea of a possible penetration of the buffer components into the cell has been considered in connection with the effects of the fermentation solutions or microbial growth mediums in general. This is understandable bearing in mind that the use of buffers has developed parallelly with enzymological researches, the role of the cell structure being consequently pointless. Work with living cells changes the situation. The facts given above are sufficient for the conclusion that among the commonly used buffers there are systems which are either able to buffer solely the cell-surrounding solution—and the surface of the cells—or to affect, as acetic acid–acetate or ammonia ammonium chloride buffer, the acidity inside the cells as well. Since now, as in general [5], only one of the two components of the buffer, either the free acid or the base, penetrates into the cell, the interior of the cell is "buffered" otherwise than the external solution. True, even with a salt, it is feasible to buffer the cell interior. Thus for instance, with ammonium acetate both components of the dissociating salt—being at equilibrium with the undissociated ammonia and acetic acid—are able to penetrate separately into the cell (cf. [5]).

It is clear that we are here concerned with a phenomenon which is not restricted to microbial cells alone but has a general cell physiological significance. Nor is it merely a question of the buffer effect of certain substances but also of the ability of these substances to function as a substrate—or an inhibitor—in metabolic reactions. What the realization of this means for the interpretation of the earlier results or how wide a significance it will have in the development of experimental techniques, remains to be seen. However, it must be stated already in this connection that the earlier results with acetic acid-acetate buffer, which differed essentially from other results [8, 10, 12], can now be explained.

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THE FEULGEN REACTION IN MATURE UNFERTILIZED SEA URCHIN EGG[1]

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Marshak and co-workers [2, 3, 5] have postulated that the genetic material does not necessarily contain desoxyribonucleic acid (DNA). One of their most important pieces of evidence is that DNA, as demonstrated by the Feulgen nucleal reaction, is apparently absent from the nucleus in mature, unfertilized sea urchin eggs. Recently, they [4] obtained chemically small quantities of DNA from such eggs, but concluded that this DNA came from the attached polar body and syncytial cells.

This communication reports the successful application of the Feulgen reaction to mature, unfertilized eggs of *Arbacia punctulata.*

Material and methods.—Mature eggs were obtained from *Arbacia punctulata* in July, by applying a stimulus of 6 volts at the perioral region. Eggs released after this mild stimulus were generally free of syncytial cells; a higher voltage (15 volts) or the injection of KOH into the body cavity generally caused syncytial cells to remain attached to the ova.

The eggs were collected in a beaker of sea water, and resuspended in fresh sea water. The eggs were treated by the following new method: a drop of the suspension was placed on a cover slip coated with 10 per cent aqueous solution of gelatin. The excess fluid was drained off, and the smear was fixed by immersion for 30 min. in 50 per cent neutral formalin. After fixation, the cover slip bearing the eggs was washed in 3 changes of distilled water and then immersed for 10 min. in a solution of acetone–HCl (99:1) at 60°C to remove the pigment and lipides.

[1] This study was conducted at the suggestion of Dr. Daniel Mazia at the Marine Biological Laboratory, summer 1954.

[2] Rockefeller fellow, 1953–1955.

[3] I wish to thank Dr. H. Swift for his critical advice on the interpretation of the results.

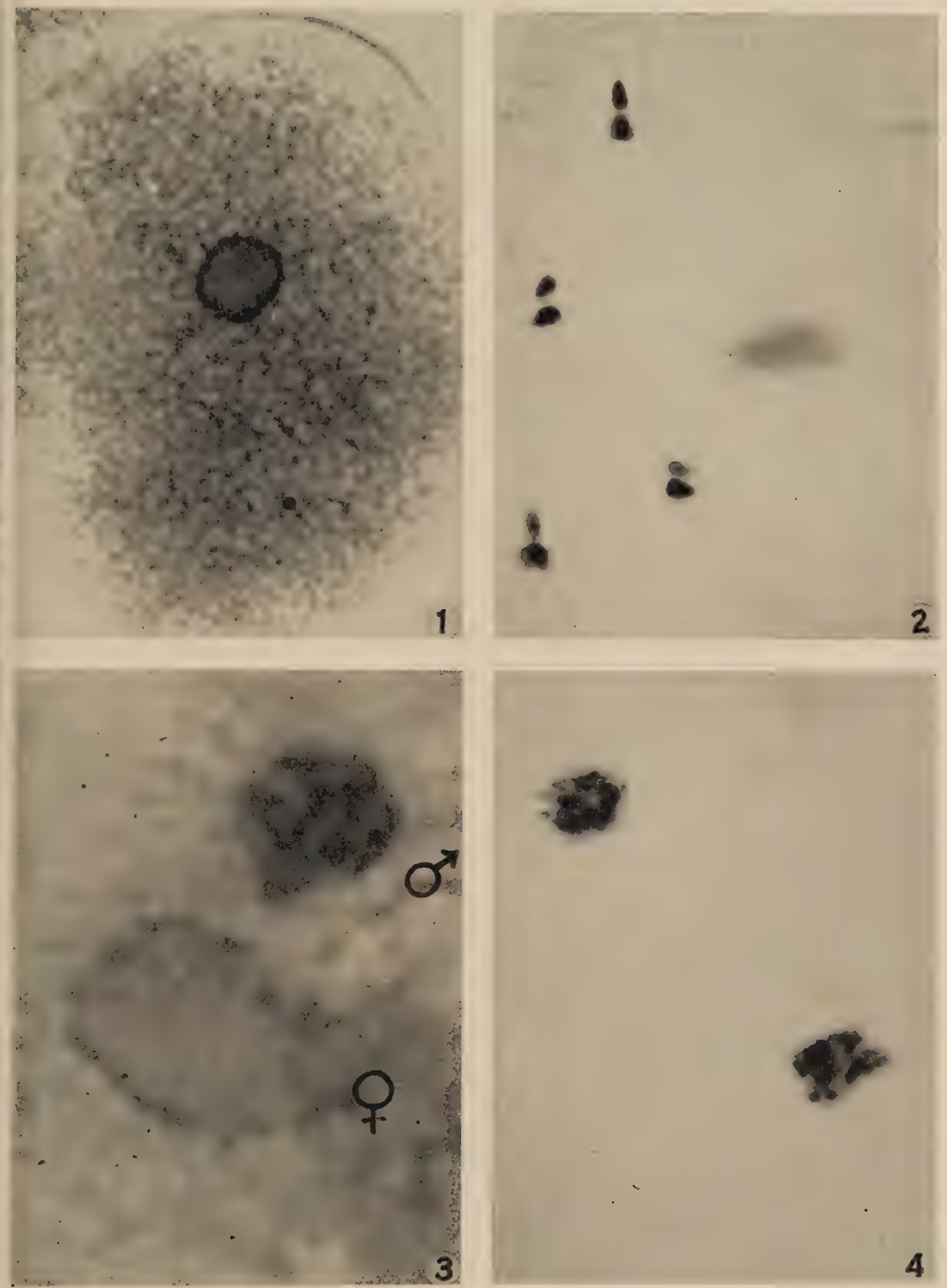
The photographs are all of sea urchin eggs (*Arbacia punctulata*) which were fixed in 50 per cent neutral formalin, extracted with 1*N* HCl at 60°C, stained for 40 minutes with the Schiff reagent and then photographed with green and yellow filters. The eggs shown in Figs. 2, 3 and 4 were treated with 10 per cent metabisulfite prior to the acid hydrolysis in order to suppress the non-specific cytoplasmic coloration which is visible in Fig. 1.

Fig. 1.—A mature unfertilized egg showing an accumulation of Feulgen reactive material on the inner aspect of the nuclear membrane. There is some non-specific cytoplasmic staining which can be blocked by metabisulfite treatment. ×1800.

Fig. 2.—An egg prepared a few minutes after exposure to a suspension of sperm. Observe the sperm heads in the jelly coat intensely stained by the Feulgen reaction. ×1800.

Fig. 3.—A fertilized egg showing the male (♂) and female (♀) pronuclei in close proximity to one another. The paler staining female pronucleus is at a slightly lower plane of focus. ×4500.

Fig. 4.—A fertilized egg in the 2 blastomere stage. The Feulgen reaction is strong positive in the chromosomes. ×1800.


1
2
♂
♀
3
4

To block any pre-existing aldehydes in the cytoplasm (plasmal?), the cover slips were immersed in 10 per cent sodium metabisulfite for 10 min. and then washed in 3 changes of distilled water. The preparations were stained by the Feulgen method [8]. Hydrolysis was carried out for 20 min., and the Schiff reaction for 40 min. The preparations were then washed in dilute sulfurous acid and distilled water, dehydrated and mounted in clarite. Controls were run without hydrolysis.

Other smears that had been fixed in Carnoy's acetic acid–alcohol (1:3) mixture were treated for 60 min. with crystalline desoxyribonuclease (DNAase, 1 mg/ml, pH 6.5) [6, 7] and then stained as above. Non-digested control preparations were also stained and examined.

In order to compare the present method with previous ones, suspensions of mature, unfertilized sea urchin eggs were fixed, treated for pigment and lipide elimination and stained by the method of Marshak and Fager [3].

Finally some eggs were centrifuged for 10 min. at 18,000 $\times g$ (Servall SS ultracentrifuge). The tube contained 2 ml of 1.1M aqueous sucrose, on which was layered 2 ml of sea water containing the eggs. After centrifugation, 3 layers appeared: an upper layer containing the nucleated portion of the broken cells, a central layer, containing some intact eggs, and a deep layer containing the bulk of the pigmented cytoplasm of the disrupted eggs. Nucleated fragments obtained from the top layer were mounted, fixed in formalin or Carnoy's mixture, and stained as above.

Other eggs obtained in the same way, were mixed with sea urchin sperm and were studied during different stages of fertilization. They were mounted, fixed and stained as described above.

Results and comment. —The eggs treated according to the method of Marshak and Fager [3] were negative by the Feulgen reaction as they reported. In contrast to their negative results, eggs treated by the present method displayed a ring of positively-stained granules around the inside of the nuclear membrane (Fig. 1). The nuclear reaction was more intense after formalin than after Carnoy's fixation [9]. The nuclei were unstained in the control preparations which were not hydrolyzed. In the preparations which were first digested in DNAase, the nuclei were also unstained. The nuclei isolated by centrifugation displayed a positive Feulgen reaction, consisting of granules attached to the inside of the nuclear membrane. However, in some eggs, these granules were displaced to one side of the nucleus, as described by Brachet [1].

Preparations made a few minutes after mixing sperm and eggs showed intensely Feulgen positive sperm heads (Fig. 2) within the jelly coat, on the surface of the egg. Figs. 3 and 4 illustrate fertilized eggs in the pronucleus phase and at the 2 blastomeres stages, respectively.

In preparations which were not exposed to sodium metabisulfite prior to hydrolysis, some degree of cytoplasmic staining occurred (compare Figs. 1 and 2). In all likelihood, this cytoplasmic reactivity is attributable to an aldehyde (lipide?) which is not extracted by the acetone-HCl. This possibility might be tested further by using other organic solvents, such as chloroform and methanol instead of acetone–HCl.

The concept that desoxyribonucleic acid is an important component of chromosomes is accepted by a majority of the workers in this field [1].

Recently, Marshak and Fager [3] and Marshak and Marshak [5] reported that there was an absence of DNA, as revealed by the Feulgen reaction in the nuclei of

mature unfertilized eggs. These investigators interpreted their data as indicating that DNA was not a necessary constituent of chromosomes.

The present observations indicate clearly that there is a detectable quantity of DNA in the nuclei of the mature, unfertilized sea urchin egg. Therefore this cell cannot be considered to be an exception to the ideas that chromosomes are composed principally of DNA-protein and that DNA is an integral part of the structure of the gene.

It seems probable that the negative reactions of mature unfertilized eggs obtained by Marshak and Fager [3] resulted from the more prolonged treatment which they employed to remove the pigment and lipides. Their results [3, 4] might be explained also on the supposition that the nuclei in the germinal vesicle stage are more sensitive to DNA extraction than are those of oogonia and young oocytes.

Summary.—A positive Feulgen reaction has been demonstrated in the nuclei of mature, unfertilized sea urchin eggs *Arbacia punctulata*, after removal of pigment and lipides by treatment with acetone HCl at 60°C. Nuclei of the nucleated fragments isolated by centrifugation also gave a positive reaction. The nuclei were negative in eggs that had been hydrolyzed or that had been treated with DNAase. A non-specific cytoplasmic reaction was present in formalin-fixed eggs, but was absent following treatment with sodium metabisulfite before hydrolysis.

The evidence presented here reaffirms the widely accepted theory that DNA is an important component of the gene.

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THE CONTRIBUTION OF EPIDERMIS AND DERMIS TO THE RESPIRATION OF THE EAR SKIN OF THE GUINEA-PIG, RAT AND MOUSE

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In the course of previous work [3, 4] it was found that thin slices of guinea-pig skin respired at rates considerably higher than those reported for other mammals. This was attributed to the fact that the guinea-pig ear skin slices comprised a thick

cellular epidermis with only a fine supporting framework of dermis. It is generally assumed that the respiration of the dermis is low and that of the epidermis is high but no measurements of their relative contribution to the respiration of skin *in vitro* appear to have been made. Knowledge of this factor is of some considerable importance in respiration studies of skin. It is conceivable that stimulant or toxic substances may have a selective action upon epidermis or dermis which might be masked by the preponderance of one or other components. Variation in the absolute values for the respiration of skin slices may be caused by variation in the thickness of the slice used, not only by virtue of the effect on oxygen diffusion but also because of the considerable differences between the respiration of epidermis and dermis. The contribution of the former will remain constant while that of the latter will vary according to thickness. Accordingly measurements have been made of the respiratory rate of separated epidermis and dermis from the ears of guinea-pigs, rats and mice.

Materials and methods.—Adult white guinea-pigs and white rats of about 800 and 250 grams respectively were used. Only mixed strains of mice were available. The slices were cut free-hand from the dorsal surfaces of the ears with a safety razor blade which had been snapped in two longitudinally. It was found advantageous in dealing with the rats and mice to attach a portion of "Sellotape" to the under-surface of the ear to facilitate holding it. The slices were between 0.1 and 0.2 mm thick.

Separation of epidermis from dermis was accomplished by incubation in trypsin solution (50 mg/ml in Ringer bicarbonate) after the method of Medawar [5]. 15 minutes' incubation was ample to permit stripping of the thin slices and did not interfere unduly with respiration.

The respiration was measured in the differential micro-respirometer described by Cruickshank [3] in a medium consisting of homologous serum 5 parts, Krebs-Ringer phosphate 3 parts, streptomycin 500 μg/ml 1 part, 5 per cent glucose 1 part. While the skin and epidermal slices floated on the surface of the medium, it was found necessary to support the dermis by means of a platform of tantalum gauze covered with lens tissue—a device used by Trowell [6] for the cultivation of lymph nodes. Measurements were made over a period of 2 hours in air.

In each experiment four respirometers were used containing respectively (1) normal control slices; (2) slices incubated with trypsin but not separated; (3) epidermis; (4) dermis. Because of the difficulty of handling small portions of epidermis the results are expressed in terms of final dry weight, about 3 mg being used in each respirometer. Six experiments were made on each species.

Results.—The results are shown in Table I. The control slices of guinea-pig skin respired at a rate of 3.60 μl/mg/hr. Treatment with trypsin however caused a slight increase in respiration to 4.3 μl/mg/hr. The separated epidermis respired at 5.29 μl/mg/hr. and the dermis at 2.21 μl/mg/hr. The mean ratio by weight of epidermis to dermis in the skin slices which were separated was found to be 2.0/1. Thus, as epidermis respires at more than twice the rate of dermis and is present in the proportion of 2/1 it must contribute more than 80 per cent of the respiration of the intact slice. A further interesting comparison may be made by calculating the hypothetical respiration rate of a skin slice using the figures obtained separately for epidermis and dermis and combining them in the proportion of 2/1. In this way epidermis and dermis would show a respiratory rate for the hypothetical slice of 4.26 μl/mg/hr.—a rate very close to that of trypsin-treated guinea-pig ear skin.

The control slices of rat skin respired at a rate of 1.93 μl/mg/hr. Trypsin treatment without separation caused in this instance a slight fall in respiration to 1.69 μl/mg/hr. Separated rat epidermis respired at 3.69 μl/mg/hr. and the dermis at 0.90 μl/mg/hr. The mean ratio by weight of epidermis to dermis was 0.3/1, about three times as much dermis as epidermis being present. Accordingly in the intact rat ear slice the epidermis contributed only just over half of the total respiration. The hypothetical combined respiration of epidermis and dermis, calculated as previously, gives a figure of 1.72, which again is close to that of trypsin-treated skin.

TABLE I

The respiratory rates of skin, epidermis and dermis from guinea-pig, rat and mouse ear.

	Source of skin					
	Guinea-pig	S.E. of mean	Rat	S.E. of mean	Mouse	S.E. of mean
Normal QO_2	3.60	$\pm$0.12	1.93	$\pm$0.12	2.56	$\pm$0.29
QO_2 after trypsin	4.30	$\pm$0.14	1.69	$\pm$0.17	2.26	$\pm$0.12
QO_2 of epidermis	5.29	$\pm$0.36	3.69	$\pm$0.21	2.95	$\pm$0.33
QO_2 of dermis	2.21	$\pm$0.32	0.90	$\pm$0.20	1.40	$\pm$0.14
Ratio by weight of epidermis/dermis	2.0/1		0.3/1		1.4/1	

Slices of mouse skin respired at a rate of 2.56 μl/mg/hr. under control conditions and after trypsin treatment at 2.26 μl/mg/hr. Measured separately the respiratory rates of epidermis and dermis were respectively 2.95 μl/mg/hr. and 1.40 μl/mg/hr. In this instance the mean ratio of epidermis/dermis was 1.4/1 and consequently the hypothetical respiratory rate of epidermis and dermis combined in that proportion is 2.31 μl/mg/ hr.

Discussion.—Our value for the QO_2 of guinea-pig ear skin is similar to that previously reported ([3] Cruickshank, 1954). The values for the QO_2 of mouse and rat skin are higher than those of other workers. Bullough and Johnson [2] give a figure of 1.18 μl/mg/hr. in air for mouse ear "shavings" and Barron *et al.* [1] state that rat skin respires at 1.0 μl/mg/hr. The latter workers used considerably thicker slices of body skin. Of more interest, however, are the respiratory rates of separated epidermis and dermis. The differences in respiration rates between the epidermis of the three species emphasise that even epidermis is not a uniform tissue. While the guinea-pig epidermis comprises 6–12 layers of active epidermal cells beneath the keratin and horn cells, the mouse possesses only a single basal layer and the rat three layers at the most. Thus the proportion of active cells by weight of epidermis varies considerably. The respiration of the dermis also shows a variation from 2.21 μl/mg/hr. in the guinea-pig to 0.9 μl/mg/hr. in the rat and this appears from histological observation to be associated with a difference in cellularity and the number of fine blood vessels and capillaries present. It should be noted that the exceedingly thin slices used, while easily obtained from the guinea-pig, could only be cut with difficulty

from the rat and mouse. Knowledge of the respiratory rate of the separated epidermis and dermis makes it possible however to assess to what extent respiration in thicker preparations may be reasonably assumed to be due to epidermal activity.

Summary.—The respiration rates of skin slices, separated epidermis and dermis from the ears of guinea-pigs, rats and mice have been measured. The results are discussed in relation to differences between the species, and the general problem of determining the QO_2 of skin.

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THE ACTION OF UREA ON SOME PROTEINS OF THE UNFERTILIZED AND FERTILIZED SEA URCHIN EGG[1]

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It has recently been observed [2] that a protein fraction precipitated at 50 per cent saturation of ammonium sulphate from an extract at low molarity (Borate buffer 0.05*M*, pH 8.2) of sea urchin eggs (called fraction I) undergoes some change at fertilization as a result of which it becomes (a) less sensitive to trypsin and (b) less precipitable by an acid buffer at high molarity [1] both in the cold and after denaturation. To account for these changes two alternative interpretations were presented: either a splitting of a complex present in the unfertilized egg or a change of configuration of at least a group of protein molecules of fraction I as a result of fertilization. It appeared then to be of considerable interest to investigate further the nature of these changes. In this paper some evidence is given for the re-arrangement of the structure of the protein molecules of this fraction upon fertilization.

In this investigation the extraction procedure has been somewhat changed with respect to that used previously as a solution of higher molarity has been employed (a buffered 1*M* solution of LiCl, see Experimental). Ranzi and Citterio [7], found in fact that this is the best extracting medium for proteins. Furthermore, versene has been added to the solution in order to prevent the action of ions which, like Ca^{++}, are known to exert a considerable influence on the proteins of the sea urchin egg [4]. The whole fraction precipitated from the extracts at 50 per cent saturation of am-

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TABLE I

Viscosity (η_{sp}) change in fraction Ia from unfertilized and fertilized eggs as a result of treatment with urea.

Unfertilized		Fertilized	
Control	Treated	Control	Treated
1.10	1.11 (+0.9 %)	0.51	0.58 (+13.7 %)
0.34	0.40 (+17.6 %)	0.30	0.36 (+20 %)
0.72	0.84 (+16.7 %)	0.36	0.51 (+41.6 %)
0.54	0.75 (+39 %)	0.39	0.66 (+69.2 %)
0.28	0.36 (+28.5 %)	0.25	0.32 (+28 %)
0.23	0.28 (+22 %)	0.15	0.21 (+40 %)
0.38	0.50 (+31.6 %)	0.16	0.22 (+38 %)

TABLE II

Increase of the intensity of the Folin reaction (Filter 66) after urea treatment of fraction Ia from unfertilized and fertilized eggs.

Unfertilized		Fertilized	
Control	Treated	Control	Treated
151	177 (+17 %)	76	100 (+31 %)
137	169 (+22 %)	75	94 (+27 %)
200	227 (+13.5 %)	255	340 (+33 %)
254	276 (+8.7 %)	280	355 (+27 %)
228	297 (+30.2 %)	220	315 (+43 %)
72	106 (+47 %)	106	154 (+51 %)
275	340 (+23 %)	145	215 (+45 %)

monium sulphate (called fraction Ia) has been studied. This fraction does not consist of a single family of protein molecules: electrophoretic analysis indicates in fact three main components (D'Amelio, in press).

Should, according to the above-mentioned alternative, the shape of the particles of fraction Ia vary somewhat upon fertilization, one would expect to find a difference in the intrinsic viscosity between the fraction prepared from unfertilized and the one prepared from fertilized eggs. However, as shown by the data plotted in the graph Fig. 1, no such difference has been found. On the other hand, as these observations have been made not on a pure protein but on a mixture of at least three different proteins, they cannot be considered as a definite answer to the problem. Rather small changes of one component only could indeed easily escape observation.

Differences between the two fractions, the one from unfertilized and the other from fertilized eggs, have been revealed by the study of the effect of urea. A 6*M* urea

solution causes a precipitate in the solution of the fraction prepared from unfertilized eggs whereas none appears in the one from fertilized eggs.

Clear-cut results have been obtained instead, using lower concentrations of urea according to the technique of Ranzi and Citterio [6].

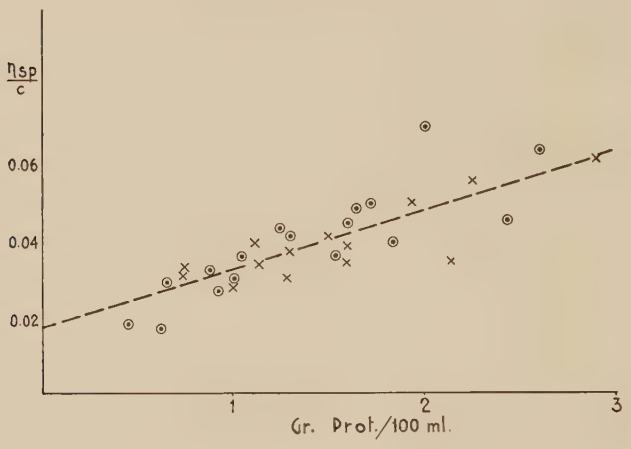

Fig. 1. Diagram for the determination of the intrinsic viscosity of fraction Ia from unfertilized and from fertilized eggs. In the abscissa the concentration of the solutions in g of protein/100 and in the ordinate the values of the ratio specific viscosity : concentration.

Under these conditions an increase of viscosity of both fractions has been found to occur (Table I); however, except for one case, this was consistently greater in the fraction prepared from fertilized eggs. This shows that the molecules of the latter undergo a greater unfolding as a result of urea denaturation.

Moreover, since denaturation causes an increase of titrable phenolic groups [3], these were estimated in fraction Ia before and after urea denaturation. As shown by the data in Table II, here again the increase is far greater in the fraction prepared from fertilized eggs.

These results are suggestive of an internal re-arrangement of a group of protein molecules without, however, any appreciable change of their shape. It appears as if H-bonds were more exposed to the attack of urea in the fraction from fertilized eggs, i.e. the bonds are broken down more easily in this latter, hence resulting in a greater unfolding of the molecules. The new configuration appears also to make these molecules less susceptible to the attack of trypsin and to heat denaturation [2]. Although it is quite possible that as a part of this process masking groups are split off, this cannot be decided on the basis of the present experiments.

Experimental.—Eggs and sperm of *Arbacia lixula* were obtained by spontaneous shedding after cutting the shells. The eggs were washed with several changes of sea water and the jellycoat removed with sea water acidified to about pH 5. Fertilized eggs had their jellycoat removed just prior to collection, i.e. about 15 minutes after fertilization.

The jelly-free eggs were rapidly collected with a gentle centrifugation and homogenized in the cold with about ten volumes of the following solution: LiCl 1*M*, $NaHCO_3$ 0.04*M*, Na_2CO_3 0.01*M*, versene 0.01*M*.

The homogenates were centrifuged 30 minutes at 20,000 × *g* in the cold using the high-speed attachment of the International Refrigerated centrifuge. The supernatant

was collected carefully and precipitated in the cold by the addition of an equal amount of buffered (0.007*M* final concentration of phosphate buffer pH 7.1) saturated solution of ammonium sulphate. After standing in the cold for about 1 hour, the precipitate was centrifuged in the cold as above and redissolved in 0.5*M* KCl in 0.035*M* phosphate buffer pH 7.1.

For the viscosimetric assay of the effect of urea, to 3.0 cc of the above solution of the fraction, 1.0 cc of a 30 per cent solution of urea was added and, after standing overnight in the cold, the viscosity was determined in an Ostwald viscosimeter at 20.5°C. As a control, equal amounts of the solution of the fraction were used to which 1.0 cc of the KCl solution was added. In some experiments even smaller quantities of urea were used, as actually suggested by Ranzi and Citterio [6]. Titrable phenolic groups were estimated by the method of Haurowitz and Tekman [3]. Protein concentrations were determined by the method of Lowry *et al.* [5] and with a standard of crystalline serum albumin as a reference.

Summary.—The action of urea on the protein fraction precipitated at 50 per cent saturation of ammonium sulphate from unfertilized and fertilized eggs of *Arbacia lixula* has been investigated. It is found that the fraction prepared from fertilized eggs undergoes a greater unfolding than the one prepared from unfertilized eggs. This, together with the results of estimation of exposed phenolic groups and of intrinsic viscosity, suggests that fertilization brings about an internal re-arrangement of the protein molecules of this fraction.

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THE EFFECTS OF SOME PURINES AND PURINE-ANALOGUES ON ASCIDIAN EMBRYOS

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A number of authors have recently found that purines and purine-analogues may produce considerable effects, usually of an inhibitory nature, on the development of vertebrate embryos; and in some cases these effects are relatively tissue-specific

[1, 2, 3, 4]. These substances may be expected to interfere, in the first place, with nucleic acid metabolism, and from this secondary consequences on protein metabolism may follow. It seemed of some interest to test their action on *Ascidian* eggs, which differ from the amphibian and avian material which has been studied previously in being of a much more "mosaic" type in their developmental mechanics. A short series of experiments was made at the Biological Station, Naples. One of us (C. H. W.) would like to express gratitude to the University of Edinburgh and the Agricultural Research Council for financial support.

The material used was *Phallusia mammilata*, and the substances tested were the natural purines adenine and hypoxanthine and the purine analogues benzimidazole and 8-azaguanine. Three main experiments were made. In each of these, a considerable number of artificially fertilised eggs, at the stage of the first or second cleavage, and still in their follicles, were put into Stender dishes containing either normal sea water (as controls) or a series of concentrations of the purines dissolved in sea water. The results were as follows:—

Adenine.—At concentrations of 0.05 and 0.025 per cent adenine caused a marked inhibition, development being arrested at early cleavage stages, or at best at the blastula. Sections of such embryos showed many lobed nuclei, or cells containing two or more nuclei. The latter appearance suggests that cells either fail to separate, or that fusion of blastomeres occurs. In cleavage stages of normal eggs, the nuclei are surrounded by haloes of darkly staining cytoplasm; these were absent from the treated material. At 0.01 per cent the effect had almost disappeared, and embryos sometimes developed as well as the controls.

Hypoxanthine.—This substance rather surprisingly acted in a stimulating manner, the treated embryos developing faster, and appearing more healthy, than the controls. This effect was noticeable both at 0.05 and 0.025 per cent, which were the only concentrations used. Thus in one experiment, in which the controls after 22 hours (at about 15°C) had developed into tadpoles just prior to the appearance of the eye-spots, the hypoxanthine embryos had well developed eye-spots and longer tails.

Benzimidazole.—Arrest of development in the cleavage stages occurred at concentrations down to 0.005 per cent. In some embryos kept in 0.001 per cent, development proceeded to the larval stage, but at a slightly slower rate than in the controls.

8-Azaguanine.—At 0.01 and 0.005 per cent this substance had comparatively slight and rather variable effects. In most cases, development was somewhat slower than normal, but in one experiment it was markedly inhibited, the embryos remaining at a neurula stage when the controls had already acquired considerable tails though no eye-spots; in another case (at 0.01 per cent) development of some embryos was rather better than controls, many of the embryos having single eye-spots when the controls had none (and the hypoxanthine embryos had two); but in the same dish some embryos were inhibited.

Combinations of substances.—The inhibitory effects of benzimidazole at 0.005 per cent were not relieved, and if anything exaggerated, by the simultaneous application of the naturally occurring purine adenine at 0.025 per cent. This is not surprising, since as we have seen the latter substance is itself inhibitory. Similarly a combination of adenine at 0.025 per cent with 8-azaguanine at 0.005 per cent proved considerably more harmful than the latter substance by itself. On the other hand, the other natural purine tested, hypoxanthine at 0.025 per cent, which is stimulating by itself, also

caused a stimulation of development when combined with adenine at 0.01 per cent or 8-azaguanine at 0.005 per cent. These are concentrations at which adenine and 8-azaguanine are almost losing their effectiveness, though they are still slightly inhibitory; the fact that the stimulatory action of hypoxanthine was not affected by them suggests that there is a true competitive interaction. Unfortunately hypoxanthine was not tested with higher concentrations of adenine and 8-azaguanine. It seems probable, on the other hand, that hypoxanthine does not enter into competition with benzimidazole, since when the latter substance at 0.005 per cent (at which it is still clearly deleterious) is combined with hypoxanthine at 0.025 per cent, the harmful action of the benzimidazole is scarcely alleviated, the embryos being arrested in cleavage or early blastula stages.

These results make it clear that *Phallusia* eggs are sensitive to the presence of purines and similar substances down to concentrations of as little as 0.01 or 0.005 per cent. The effects of the substances cannot be easily deduced *a priori*, since one of the natural purines (adenine) and one of the "purine antagonists" (8-azaguanine) are harmful, while the other natural purine (hypoxanthine) actually stimulates development, and the other antagonist (benzimidazole) is strongly deleterious.

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ELECTRON MICROSCOPY OF COLLAGEN AND ELASTIN IN LAMINA PROPRIA OF THE TRACHEAL MUCOSA OF RAT

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Electron microscopy of connective tissue has mainly been performed with the aid of fragmentation and shadow casting methods, some of the work of extremely high quality [1, 8, 9, 10, 13, 14, 16]. The sectioning technique has so far been used only in a few cases [3, 12], the results being moderate. When analyzing the tracheal mucosa of rat [4, 5, 6] the lamina propria turned out to be of interest representing a good field for studying connective tissue. In this preliminary report, the characteristics of collagen and elastin as seen in sections will be dealt with shortly.

The tissue was fixed in isotonic osmium tetroxide solution, buffered to pH 7.2 with acetate-veronal, and embedded in a mixture of 95 per cent butyl methacrylate and 5 per cent methyl methacrylate, containing by weight 0.2 per cent benzoyl peroxide.

After polymerization the tissue was sectioned with a Sjöstrand-Ultramicrotome and analyzed in an RCA EMU 2c electron microscope.

The main components of the lamina propria are the fibroblasts, the collagen, and the elastin fibers. The fibroblasts are elongated cells with a well-defined cell membrane, 80 ± 2 Å ($\delta = 12$) thick. The nucleus is lobated, the cytoplasm finely granulated (150 ± 2 Å; $\delta - 10$) with very small mitochondria, 0.2 μ in width, and a Golgi region as previously described in other kinds of cells [7, 11].

The collagen fibrils are aggregated in bundles with 1000–2000 in each. They are well separated from the fibroblast by its cell membrane. In longitudinal section the diameter of the collagen fibril is 520 ± 7 Å ($\delta = 36$) and in transverse section 690 ± 15 Å ($\delta = 81$). The periodicity of the transverse bandings is 665 ± 3 Å ($\delta = 14$). Each period consists of 4 osmiophilic bands, *a*, *b*, *c*, and *d* (using the denomination of Schmitt and Gross [8]) with an approximate thickness of the single band of 85 Å. The less osmiophilic space between *a* and *b*, *c* and *d* equals and is 65 Å, that between *b* and *c* 90 Å and between *d* and *a* 170 Å. In longitudinal sections, the indication of an outer limiting membrane is vague, being more pronounced in transverse sections. Its thickness is around 60 Å.

The elastin occurs in dense fibers with a diameter of about 1–2 μ. No branching has been noted. The fibers are composed of longitudinally arranged fibrils with a thickness of 70 ± 3 Å ($\delta = 16$). The fibrils are seen especially well at the outer part of the fiber, where they apparently are not so densely packed as in the center of the fiber. No axial periodicity has been noted in the fibrils.

The periodicity of the collagen fibril shows up well in sections, thus proving that the bands are plates which penetrate the whole fibril. The tubular structure demonstrated by Kennedy [3] cannot be observed, when leaving the plastic in. The revomal of the embedding medium certainly destroys the fine structure of the fibril. The bands observed in the present investigation are not of the same number as shown by Schmitt and Gross [8]. This may be due to the low contrast of the structures observed and to the relatively low magnification. It has not been possible to resolve the *b* band as a double band (b_1 and b_2, Schmitt and Gross [8]) and furthermore in this material the thickness of the *b* band seems to be similar to that of the *a*, *c*, and *d* band. As tho the *e* band there is only in some pictures a slight indication of a density in the space between *d* and *a* band. Using PTA solution for staining will certainly improve the contrast and be helpful in solving this discrepancy between observations made in sections and on isolated material.

As for the elastin, the fibrillar structure has for the first time been demonstrated in sections and in untreated material. After tryptic digestion of isolated material, Gross [1] found fibrils with a thickness of 70 Å. Wolpers [15], after treating the elastic tissue with pepsin in acid, claimed the thinnest fibrils to be 80 Å. Hall, Reed and Tunbridge [2] observed very fine fibrils, 200 Å thick, only in elastase-treated material. All these fibrils may correspond to the fibrils demonstrated here with a thickness of 70 Å. In this study there is no indication of the coiled fibrils found by Gross [1]. The

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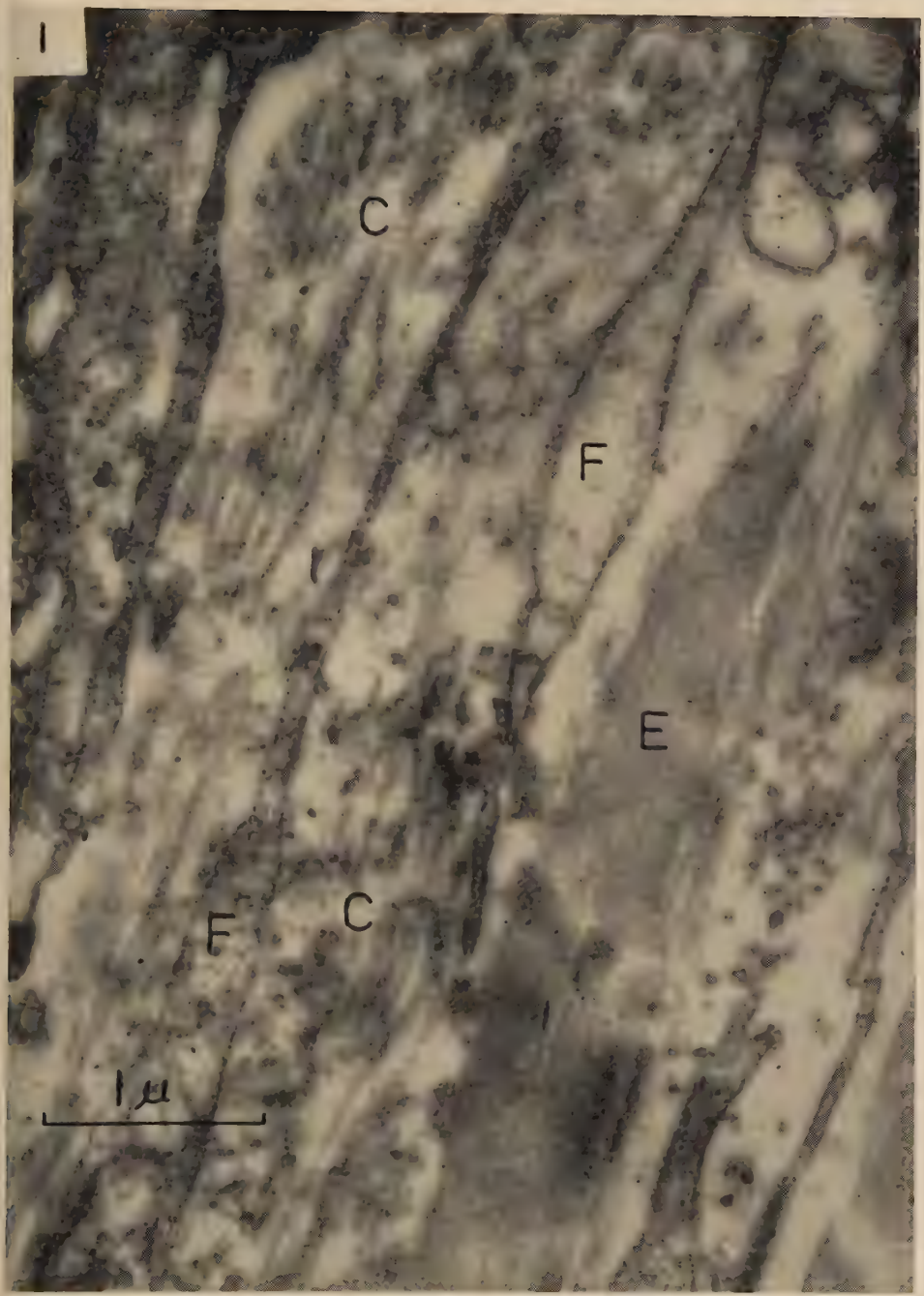
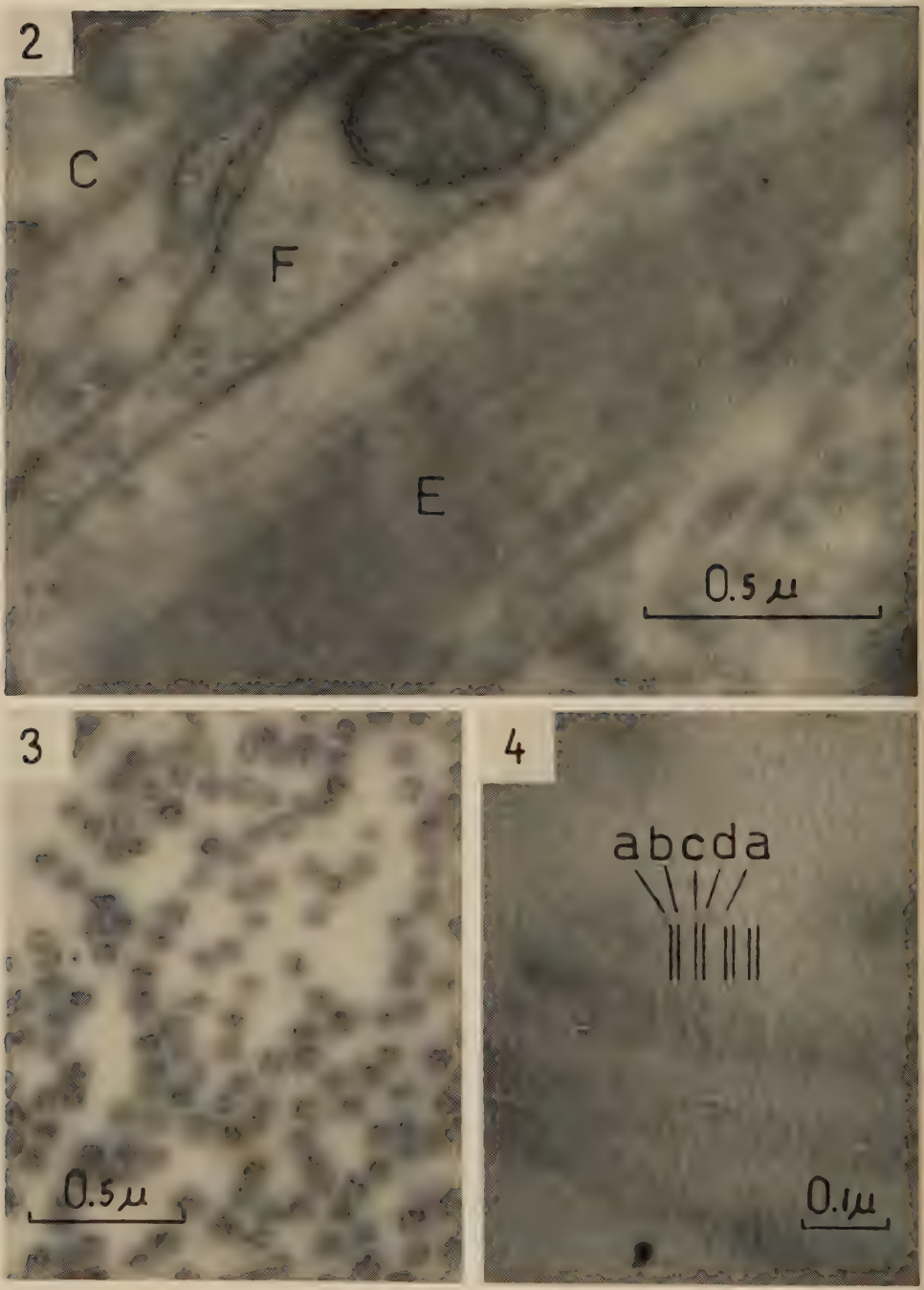
Abbreviations: C, collagen fibrils, E, elastin fiber, F, fibroblast.

Fig. 1. The connective tissue of lamina propria is arranged in layers where the collagen fibrils, elastic fibers and fibroblasts form the constituents. Magnification × 30,000.


1
C
F
E
F
C
1 μ


2
C
F
E
0.5 μ
3
0.5 μ
4
abcda
0.1 μ

present findings are more in agreement with the assumption by Hall, Reed and Tunbridge [2], viz., that the elastic fibers consist of at least two components, fine fibrils and a dense, non-fibrous cement material.

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Fig. 2. Upper left corner two collagen fibrils with the typical transverse bandings. At F part of a fibroblast. Note the distinct cell membrane. In the cytoplasm a mitochondrion with the characteristic features of this organell. In the middle an elastic fiber with a dense central part. At the outer parts the elastic fibrils, 60 Å thick, can be seen. Magnification × 66,000.

Fig. 3. Transversely cut collagen fibrils. The outer limiting membrane, 60 Å thick, stands out most clearly when the fibrils are sectioned through a bright interspace between the dense bands. Magnification × 43,000.

Fig. 4. High power view of longitudinally cut collagen fibrils. When fixed in osmium tetroxide the contrast is poor. However, the main periodicity can be seen, clearly demonstrating that the dense bands penetrate the whole fibril. Magnification × 117,000.

OBSERVATIONS ON THE CYTOCHROMES OF *AMOEBA PROTEUS*, *CHAOS CHAOS* AND *TETRAHYMENA GELEII*

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Although the respiration of *Amoeba proteus* [4] and *Chaos chaos* (*Pelomyxa carolinensis*) [10, 11] is largely inhibited by cyanide, no direct evidence of the presence of the cytochrome system in these organisms has been reported. On the contrary, using a very sensitive method, Andresen *et al.* [2] were unable to detect any cytochrome *c* oxidase in *Chaos chaos* with commercial cytochrome *c* as substrate. Repeated attempts to demonstrate cytochrome *c* oxidase in *Chaos chaos* spectrophotometrically

have failed [7], again using commercial cytochrome *c* as substrate. On the other hand, succinic dehydrogenase [2] has been demonstrated in *Chaos chaos*, indirectly implying the presence of cytochrome *b*.

The principal obstacle in studying the cytochrome system in *Chaos chaos* and *Amoeba proteus* has been the difficulty of obtaining enough organisms for even micro-scale work from the usual hay and wheat infusion type of culture [1]. Using the method of Prescott and James [14] for *Amoeba proteus* and a slight modification of this method for *Chaos chaos* [13] large amounts of clean amoebae (10 to 20 cc of packed cells) can be obtained without difficulty. We were thus able to search for the cytochrome system by direct microspectroscopic observations (Zeiss Mikro-Spektral-okular, Abbe).

Several cubic centimeters of starved and washed *Amoeba proteus* or *Chaos chaos* were broken up by freezing, the preparation reduced with dithionite ($Na_2S_2O_4$), and observed in the frozen state ($-10°C$). Because the amoebae were grown exclusively on *Tetrahymena geleii* (GL), on organism which contains cytochromes, the amoebae had to be well starved before use. The amoebae were usually starved at 23°C for four days, which is one day in excess of the time necessary for clearing out of all food vacuole traces. In a test experiment in which 2 cc of *Amoeba proteus* were starved for 12 days, the cytochrome bands (see below) were not only undiminished in strength but on the contrary appeared somewhat intensified. Under these conditions cytochrome absorption bands in the visible spectrum were seen at the following wave lengths: 553, 562, 605, 522 and 531 mμ in order of decreasing intensity. The 605 mμ band in *Chaos chaos* was somewhat weaker per volume of packed cells than in *Amoeba proteus*. Cooling reduced amoeba homogenates containing 50 per cent glycerol (v/v) to the temperature of liquid nitrogen ($-196°C$) according to the procedure of Keilin and Hartree [9] did not sharpen or intensify the absorption bands but on the contrary blurred the whole spectrum.

The absorption lines at 562 and 531 mμ are probably due to cytochrome *b*. The 553 and 522 mμ bands we ascribe to cytochrome *e* (see below) and the 605 mμ line is probably due to cytochrome *a* or cytochrome *e* oxidase or both. If the homogenate is kept at 0° to $-10°C$ the compounds responsible for the absorption lines are quite stable and can be repeatedly oxidized and reduced. Oxidized homogenates showed no detectable absorption lines. None of the cytochromes could be brought into solution using the following procedures: (1) the Keilin and Hartree method for beef heart cytochrome *c* [8], (2) treatment with 4, 8, or 12 per cent sodium desoxycholate at 23°C for up to 24 hours, (3) autolysis at 23°C for one hour. Following the two last treatments the absorption bands were still to be seen in the precipitate. The absorption bands at 553 and 522 mμ [9, 12] and the insolubility of the compound responsible for these absorptions [6, 12] strongly indicates the presence of cytochrome *e* (called cytochrome b_5 in [12]). No cytochrome *c* could be detected.

The position of cytochrome *e* in the oxidation reduction chain has not yet been established, although there is some evidence that it acts in an oxidation pathway independent of at least part of the system containing cytochrome *c*, and it does appear to possess its own specific terminal oxidase [3]. These two points are supported by what is known from studies on *Amoeba proteus* and *Chaos chaos:* (1) these amoebae contain cytochrome *e* but no detectable cytochrome *c*, (2) cytochrome *c* oxidase has not been detected in these amoebae using highly sensitive methods [2], and (3) the

respiration of these amoebae is strongly inhibited by cyanide [4, 10, 11] indicating the presence of a terminal cytochrome oxidase.

From our own studies on *Tetrahymena geleii* (GL) and from the work of Eichel [5] it appears that these organisms also operate on a cytochrome *e* system. In washed, packed *Tetrahymena geleii*, observed spectroscopically in the manner described above for amoebae, we have observed intense absorption lines (25 mm light path) at 553 and 605 mμ and faint but distinct bands at 564, 528, and 520 mμ. We were not able to bring into solution any of these cytochromes with the methods 1 and 2 mentioned above for amoebae. By the third method (autolysis) we were able to solubilize some of the 553 mμ component. This solution measured against water in the Beckman DU spectrophotometer showed absorption maxima at 553, 522 and 415 mμ. Even in frozen homogenates ($-10°$C) the absorption bands became weaker with time, particularly at the surface of the preparation; except for the 605 mμ band all bands could be recalled with sodium dithionite. After 24 hours at $-10°$C the 605 mμ band was no longer detectable in reduced homogenates and a new, blurred absorption line had appeared at 585 to 590 mμ and another at 578 mμ. We tentatively consider the 605 mμ band to represent the terminal oxidase (cytochrome *e* oxidase?) of the DPNH oxidase system described by Eichel for *Tetrahymena geleii* [5]; Eichel reports 70 per cent loss of DPNH oxidase activity in 2 hours at 0°C.

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CYTOPLASMIC GRANULES IN MYOGENIC CELLS

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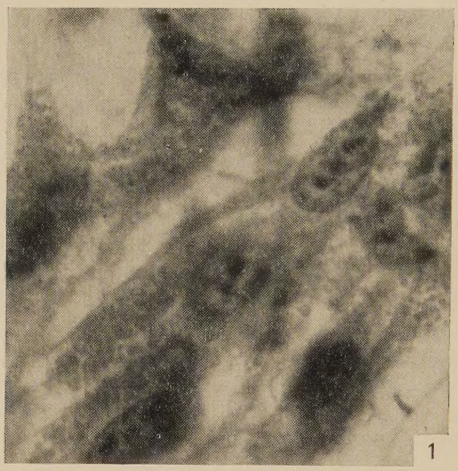
In the course of a study on the development *in vitro* of embryonic chick muscle cells and the formation of myofibrillae, characteristic granular inclusions have been observed in the cytoplasm during the early phases of cytodifferentiation. Attention



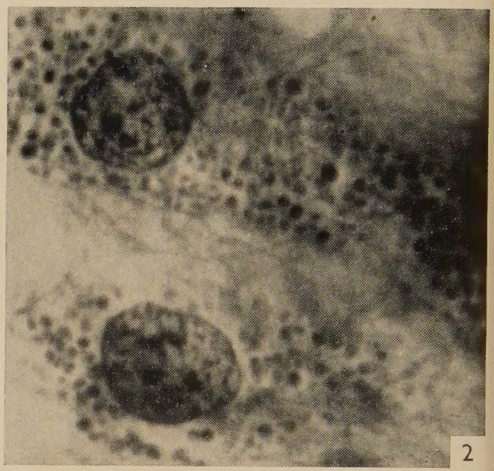


Fig. 1. Section through an outgrowth area of a 3-day culture of myogenic tissue; trapezoid-shaped and elongated myoblasts with cytoplasmic granules. Ehrlich's haematoxylin, eosine, following treatment with saliva. × 1100.
Fig. 2. Myoblasts in 2-day culture of a suspension of myogenic cells. Granules stained with Biebrich's scarlet. × 1700.

has recently been drawn to the presence of polysaccharide containing inclusions in fibroblastic cells and especially to their probable role as precursors in the elaboration of intercellular ground substance and in fibrogenesis [3, 4, 5]. On a different level of magnitude, the possibility of transmutation of globular into filamentous structures has been suggested by electron-microscopic work on the synthesis of collagen-type fibrils [12] and on fibrinogen–fibrin transformation [11]. Also, according to recent studies on nerve fibers [1], cytoplasmic microvesicular structures seem to be intimately associated with the synthesis of neurofibrillae. In this brief report the possibility is suggested that in early myoblasts comparable events may take place, namely that a sequential relationship exists between the presence and the disappearance of certain characteristic cytoplasmic granules and the elaboration of myofibrillar structures.

This possibility derives mainly from observations on the development *in vitro* of myoblasts in cultures of undifferentiated myogenic tissue from the limb-buds of 3½-day chick embryos. As described elsewhere [7, 8], the skeletogenic and the myogenic regions can be neatly separated in the skinned limb rudiment of this age and thus cultures consisting predominantly of presumptive muscle tissue can be prepared. Watch glass and hanging drop cultures were set up either with regular plasma-embryo extract clots or with bacterial cellulose membranes [9, 10] steeped, partly or entirely, in heterologous liquid media. In addition, cell suspensions were prepared by the trypsin dissociation technique [7, 8], and the discrete cells were cultivated in drops of liquid medium or in a thin film of serum in Fell's "horse-shoe" hanging drops [2]. This latter method of cultivation proved particularly commendable and enabled continuous high power observation of single myoblasts.

In addition to cultured material, histological sections of fixed and of frozen-dried embryonic tissue were examined.

Under outgrowth promoting conditions of cultivation, presumptive, non-granular myoblasts began to migrate from the tissue soon after explantation and assumed irregular, trapezoid shapes with short cytoplasmic processes. This rather distinct cell form was assumed also by the trypsin dissociated, discrete cells, soon after settling down in cell suspension cultures and, together with other characteristics, made the early myoblasts quite readily distinguishable from other cell types.

After 36 hours of cultivation some scattered myoblasts with cytoplasmic granules were already present, besides the numerous non-granular presumptive myoblasts. At this phase of cytodifferentiation the granules were relatively large, ranging from 0.8–2.2 μ in diameter. They were dispersed throughout the cell without any constant pattern of arrangement and persisted during mitosis. They reacted quite feebly and inconsistently with neutral red and did not stain supravitally with Janus green under conditions which demonstrated mitochondria. With toluidine blue the granules stained blue.

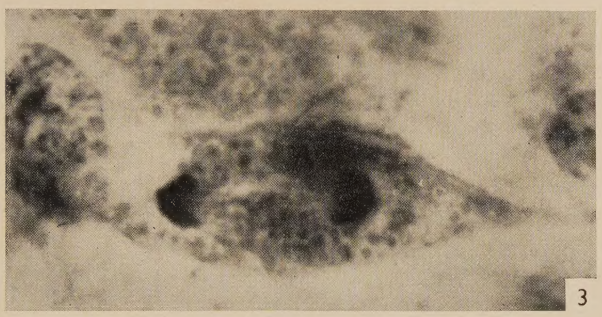


Fig. 3. Same as Fig. 2. Note persistence of myoblastic granules through late anaphase.

In sections or whole mounts of cultures and in sectioned embryonic material fixed in Helly's fluid or in formol-acetic vapour followed by chromation, the myoblastic granules stained readily with w.s. eosine, with Biebrich's scarlet and less readily with chromotrop 2R. In sections treated with alcoholic P.A.S. the granules showed a positive but faint reaction, which persisted however after extraction for lipids and treatment with saliva, and did not show without prior oxidation. Myoblastic granules thus appear to contain polysaccharide. In sections exposed for 15 min. to 1 per cent trypsin at 37°C, pH 7.8, and treated subsequently with P.A.S., the reaction of the granules appeared considerably intensified, as if additional reactive groups had become available due to the tryptic digestion. If stained with toluidine blue, the granules showed beta metachromasia. It has been assumed that the intensity of P.A.S. reaction may be directly related to the quantity of the reacting polysaccharide [4]; if so, then the above results indicate that the polysaccharide content of the myoblastic granules is relatively low and that some of it is associated with protein in a way which makes it P.A.S.-reactive only following tryptic digestion. In this respect these granules differ from the more strongly P.A.S.-reactive inclusions of fibroblasts, which apparently have a higher content of polysaccharide.

Cultured and embryonic muscle tissue fixed in cold methanol or in Sansom's modification of Carnoy's fixative was stained with methyl green–pyronine [13] and with May Grunwald–Giemsa [6]. National Aniline stains were used, the methyl-green after repeated extraction with chloroform. The myoblastic granules reacted with pyronine and stained blue with the M.G.–G. stain. Prior exposure of the sections to ribonuclease almost completely prevented these reactions. If the specifity of these staining procedures is accepted, they indicate strongly that the myoblastic granules contain ribonucleoprotein. When stained with acid dyes following ribonuclease digestion, the granules still took up the stain, due probably to the presence of acidophilic protein substances. Their size excludes the myoblastic granules from the range of the much smaller RNP containing microsomes, but they may equally represent foci of metabolic or synthetic activity.

The phase of the typical structural differentiation of the cultured myoblast was initiated by its gradual elongation, by certain changes in the structure of the interphase nucleus and by an increasing basophilia of the cytoplasm. At the same time, the myoblastic granules decreased in diameter and sometimes, especially in the more elongated parts of the cell, became arranged in bead-like strings orientated parallel to the long axis of the myoblast. The meaning of these formations is not clear, especially as during the subsequent development of the cell, the size of the myoblastic granules decreased further, until they were no longer resolvable. The disappearance of the granules was not simultaneous throughout the cell but was completed earlier in its more elongated or extended parts; it was precisely at these sites that the myofibrillar structures were first discernible. This sequence of events spread throughout the cell, the disappearance of the granules being associated with the appearance of the very delicate filamentous myofibrillae. Various intermediate stages in this process could readily be found: elongated mononuclear myoblasts were seen, one part of which still contained the granules, while elsewhere in the same cell myofibrillae had already appeared. In other cells only the fibrillae were present, the myoblast having thus entered upon a later phase of development.

Whether the sequence of events reported here actually represents a chemical or a stereochemical transformation of granular into myofibrillar material or is just incidental, cannot be profitably discussed on the basis of this brief account; our working hypothesis is that the relationship may be direct. A detailed account of these observations and of the further development *in vitro* of the myoblast will be reported later.

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